Genetic Association, Mutation Screening, and Functional Analysis of a Kozak Sequence Variant in the Metabotropic Glutamate Receptor 3 Gene in Bipolar Disorder

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Importance: Genetic markers at the gene encoding the metabotropic glutamate receptor 3 (GRM3) showed allelic association with bipolar disorder.

Objective: To screen the GRM3 gene and adjacent control regions of genomic DNA in volunteers with bipolar affective disorder for mutations increasing susceptibility to bipolar disorder.

Design: Sequencing and high-resolution melting curve analysis of DNA followed by genotyping was carried out in 1099 patients with bipolar affective disorder and 1152 healthy comparator individuals.

Setting: Participants with bipolar disorder were recruited from National Health Service psychiatric services and from patient organizations.

Participants: Individuals were included if they had Research Diagnostic Criteria diagnoses of bipolar I and bipolar II disorder and were of British or Irish ancestry.

Main Outcomes and Measures: Identification of base pair changes in the GRM3 gene that affected expression or function of the GRM3 receptor that also showed an allelic association with bipolar disorder.

Results: A base pair variant (rs148754219) was found in the Kozak sequence of exon 1 of the GRM3 gene, 2 bases before the translation start codon of one of the receptor isoforms, in 23 of 2251 people who were screened and genotyped. Nineteen of the 1099 bipolar cases (1.7%) were mutation carriers compared with 4 of 1152 healthy comparator individuals (0.3%). The variant was associated with bipolar disorder (P = .005; odds ratio, 4.20). Bioinformatic, electrophoretic mobility shift assay, and gene expression analysis found that the variant created a new transcription factor protein binding site and had a strong effect on gene transcription and translation.

Conclusions and Relevance: Confirmation of these findings is needed before the Kozak sequence variant can be accepted as a potential marker for personalized treatment of affective disorders with drugs targeting the metabotropic glutamate receptor 3.


The glutamate receptor 3 gene (GRM3; OMIM 601115) encodes the metabotropic glutamate receptor 3 (mGluR3) protein, which is a group II metabotropic glutamate neurotransmitter receptor that is similar to mGluR2. Metabotropic glutamate receptor 3 is a G protein–coupled receptor that acts by inhibition of adenylylate cyclase and reducing cyclic adenosine monophosphate production. Group II mGluRs are involved in learning, memory, anxiety, and the perception of pain. Metabotropic glutamate receptor 3 is found on presynaptic and postsynaptic neurons in synapses of the hippocampus, cerebellum, and cerebral cortex, as well as other parts of the brain and in peripheral tissues. The mGluR3 receptors modulate glutamate concentrations by sensing excess synaptic glutamate release and providing a negative feedback mechanism that downregulates glutamate transmitter release. Activation of postsynaptic mGluR2/3 receptors can reduce neuronal excitability and plasticity through intracellular mechanisms, such as modulation of ion channels and induction of long-term synaptic depression. Studies also have suggested that mGluR2/3 receptors may function as heteroreceptors controlling the release of γ-aminobutyric acid and other neurotransmitters.
The GRM3 gene has been investigated in bipolar affective disorder as part of several genome-wide association studies (GWASs) but failed to reach genome-wide significance in any of these investigations. In a collaborative study,1 15 single-nucleotide polymorphism (SNP) markers within 50 kilobases (kb) of the GRM3 gene showed allelic association with bipolar disorder. The SNP rs2237563 was the most significantly associated marker \((P = 3.85 × 10^{-5})\) in the University College London 1 (UCL1) subsample. Fourteen other markers within 125 kb of the gene also showed nominally significant associations with bipolar disorder in the UCL1 subsample. In the Psychiatric GWAS Consortium bipolar study,6 the most associated GRM3 marker was rs17161018 \((P = .00093)\). Thirty-two different SNP markers within 125 kb of GRM3 were nominally associated with bipolar disorder. Several other association studies in different populations also provided evidence of an association between GRM3 markers and bipolar disorder. In a study7 of Ashkenazi bipolar trios, GRM3 was ranked as showing “highly suggestive” transmission disequilibrium between a 4-SNP marker haplotype and bipolar disorder. A study8 of bipolar disorder in South Africa found that heterozygotes for the GRM3 SNP rs6465084 had a 4-fold risk of psychotic symptoms. A German study9 found no evidence of association between GRM3 and bipolar disorder. A study10 of major depressive disorder revealed an allelic association between the SNP rs6465084 in GRM3 and depression \((P = .037)\).

The human GRM3 gene consists of 6 exons that give rise to 4 transcript variants, with the main transcript being NM_000840. Four alternatively spliced transcripts of GRM3 have been reported11 in human brain, including a full-length transcript, a transcript with exon 2 deleted, a transcript with exons 2 and 3 deleted, and a transcript with exon 4 deleted (eFigure 1). Molecular characterization of the promoter of GRM312 provided evidence of a silencing element being present in exon 1. Clinical studies of the effect of receptor agonists and antagonists of mGluR2/mGluR3 have been carried out for the treatment of anxiety, schizophrenia, depression, and withdrawal from morphine and nicotine.13–15 We conducted a study to identify etiologic base pair changes in GRM3 that affect protein transcription and/or translation.

**UCL BIPOLAR CASE CONTROL SAMPLE**

This study included 1099 individuals with bipolar disorder, sampled in 2 cohorts. The first cohort (UCL1) comprised 506 bipolar I cases and 510 healthy comparison participants who were subjected to a GWAS resulting in publications focusing on the most statistically significant allelic associations.4,5 The second cohort (UCL2) comprised 409 bipolar I (69%) and 184 bipolar II participants. In the sample of 1152 healthy participants, 672 were screened to rule out psychiatric disorders plus 480 unscreened British healthy volunteers provided by the European Collection of Animal Cell Cultures. All cases and controls were selected to be of British or Irish ancestry. The UK National Health Service multicenter and local research ethics approvals were obtained, and signed informed consent was given by all participants. All UCL bipolar cases and the psychiatrically screened control subjects were interviewed by a psychiatrist (A.A. and others) using the lifetime version of the Schedule for Affective Disorders and Schizophrenia.6 The controls were selected if results of screening with the Schizophrenia and Affective Disorders Schedule interview were normal and it was established that they had no first-degree relatives with any mental disorder. Bipolar disorder diagnoses were confirmed in all bipolar participants according to Research Diagnostic Criteria.17 All of the bipolar participants were also rated with the 90-item Operational Criteria Checklist for Psychotic Disorders.18

Blood samples were used as the source of DNA for the cases and controls in UCL1, and saliva samples were used for the cases in UCL2. The DNA was extracted for all UCL samples using methods published previously.19

**DETECTION AND EVALUATION OF NEW VARIANTS**

The G allele of rs2237563 was the most strongly associated SNP in the UCL1 sample \((P = 3.85 × 10^{-5})\). Therefore, DNA from 32 bipolar research volunteers were selected for being homozygous or heterozygous for the G allele of rs2237563, and 32 random healthy controls were also selected for sequencing. Sequencing was carried out on the promoter region 1000 base pairs (bp) upstream of the transcriptional start site, 5' untranslated region (UTR), the exons and intron/exon junctions, and the entire 3' UTR of the GRM3 isoform 1 \((NM_000840)\), which contains all 6 exons (eTable 1; http://www.jamapsych.com). Sequencing was done (Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl DNA Analyzer; both Applied Biosystems), and sequencing data were analyzed (Staden package).20 To identify further bipolar susceptibility variants in exon 1 and 596 bp of the promoter region, high-resolution melting mutation screening using 10 primer pairs was performed in all bipolar samples. Reactions were carried out on a real-time polymerase chain reaction thermal cycler with fluorescent detection (LightCycler 480; Roche). Primer sequences and reagents are shown in eTable 1. Samples with abnormal high-resolution melting curves were then sequenced as described in the previous paragraph.

Bioinformatic analysis to determine potentially functional SNPs was carried out using the UCSC genome browser (http://genome.ucsc.edu/), PolyPhen221 and RESCUE-ESE \((http://genes.mit.edu/burgelab/rescue-ese/)\), Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess), UTRsite \((http://utrsite.ba.itb.cnr.it/)\), and Mfold \((http://mfold.rna.albany.edu/?q=mfold)\).24

**GENOTYPING AND ASSOCIATION ANALYSIS**

The SNP genotyping for the 3 most significantly associated GRM3 SNPs (rs2237563, rs274621, and rs2188786) in the UCL1 bipolar disorder GWAS was performed in the UCL2 samples by allele-specific polymerase chain reaction (KBiosciences; LGC Genomics). Rare variants or potentially etiologic SNPs found by sequencing were genotyped in-house (KASPar reagents; LGC Genomics) on a LightCycler 480 (Roche) in both the UCL1 and UCL2 samples. For all SNPs genotyped, 17% of the samples were reduplicated to detect error and confirm the reproducibility of genotypes. All data were analyzed to confirm Hardy-Weinberg equilibrium. Genotypic and allelic associations for SNPs were determined using \(\chi^2\) tests. HaploBlock tests of association were performed using Haploview.25 Significance values shown for all analyses are uncorrected for multiple testing, and a cutoff significance value of \(P < .05\) was used. A gene-wide test to assess the overall evidence for association at the GRM3 locus in the GWAS data from the UCL1 sample was performed using COMBASSOC.26

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CELL CULTURE

Two human cell lines were used for transfection experiments: HEK293 (human embryonic kidney cell line) and SH-SY5Y (neuroblastoma cell line). Both cell lines were cultured in Dulbecco modified Eagle medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin solution (Sigma-Aldrich), at 37°C with 5% carbon dioxide.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS

Nuclear protein extracts were obtained from SH-SY5Y cells as described elsewhere.27 Oligonucleotides with the wild-type (G) and the mutant (A) allele of rs148754219 were 5’ end-labeled with infrared dye DY-682 (Eurofins MWG Operon). Aliquots and the mutant (A) allele of rs148754219 were 50 fmol of labeled double-stranded oligonucleotides for binding 50 fmol of unlabeled competitor probe were used in separate tubes. Binding reactions were then resolved by electrophoresis on a 4% native polyacrylamide gel containing 50mM Tris-borate buffer. The DNA-protein complex was detected on Tris-borate buffer.

CONSTRUCTION OF RECOMBINANT LUCIFERASE CONSTRUCTS

The ability of the rs148754219 variant to modulate transcription and translation of GRM3 was tested using 5 reporter vector constructs (eFigure 2A). For clone A, exon 1 of GRM3 was cloned using polymerase chain reaction into a promoterless luciferase vector (pGL3-basic; Promega) using the Xhol and BglII restriction sites. Clone B contained a polymerase chain reaction fragment comprising 523 bp of the putative minimal promoter region and exon 1 of GRM3 cloned into the Xhol and BglII restriction sites of the pGL3-basic vector. A third construct, clone C, with exon 1 of GRM3 cloned into the Xhol and BglII restriction sites upstream of the SV-40 promoter in the pGL3-promoter vector (Promega) was generated. Clone D was prepared by cloning GRM3 exon 1 into the HindIII and Ncol restriction sites downstream of the SV-40 promoter of the pGL3-promoter vector such that the luciferase gene was in-frame for translation products initiated from the ATG of the GRM3II3 isoform. The final construct, clone E, was prepared by substituting the luciferase ATG start codon to GTG using site-directed mutagenesis to test the translational capacity of the GRM3II3 ATG codon. A mutagenesis kit (QuikChange II XL Site-Directed Mutagenesis Kit; Agilent Technologies) was used to introduce the mutant allele for the SNP rs148754219 in all the clones. The sequence and orientation of all the experimental constructs were verified by DNA sequencing.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAYS

Transfection and luciferase assay experiments were performed using HEK293 kidney and SH-SY5Y neuroblastoma cells. Twenty-four hours before transfection, cells were seeded in 24-well plates at approximately 5 x 10^4 and 1 x 10^5 cells/well for HEK293 and SH-SY5Y cells, respectively, in antibiotic-free medium. Transfection was then performed (Lipofectamine 2000; Life Technologies) according to the manufacturer’s protocol. For each well, 0.8 μg of the reporter construct was transfected, with pRL-SV40 Renilla luciferase reporter vector as a control, to determine transfection efficiency at a ratio of 50:1. The transfected cells were maintained in culture, and the growth medium was changed 4 hours after transfection. The cells were harvested 48 hours after transfection, and the activity of firefly and Renilla luciferase was measured (Dual Luciferase Reporter Assay System; Promega; and TD-20/20 n luminometer; Turner Biosystems). Transfections were performed in triplicate and repeated 3 times. Firefly luciferase luminescence was expressed as a ratio to that of the Renilla activity, and the normalized data were compared with a 2-tailed unpaired t test or Mann-Whitney test (SSPS, version 18.0; SPSS, Inc). A cutoff significance of P < .05 was used.

RESULTS

GRM3 ASSOCIATION ANALYSIS

The HEK293 and SH-SY5Y cells were seeded in 6-well plates at approximately 5 x 10^4 and 1 x 10^5 cells/well, respectively, and transfections were performed after 24 hours using 2.4 μg of clone D reporter constructs and pRL-SV40 at a ratio of 50:1 per well. Total RNA was extracted after 24 hours (Illustra RNAspin Mini RNA Isolation Kit; GE Healthcare Life Sciences) and subjected to DNase treatment (Promega) for 1 hour at 37°C to eliminate plasmid DNA, according to the manufacturer’s instructions. Two micrograms of DNase-treated RNA was used to make complementary DNA in the presence of superscript II RT (Life Technologies) according to the manufacturer’s instructions. Firefly luciferase messenger RNA (mRNA) was quantified using primers F- CTTCTGATAGGGACAAGACAA, R- AGGCCACCATTTAGCAGAC, and Universal Probe Library probe 82 (UPL; Roche) (LightCycler 480; Roche). Renilla luciferase mRNA was quantified using primers F-TGTGTCAGTTAGGGTGTGGAA, R- CACCTGGTTGCTGGC, F- TGTGTCAGTTAGGGTGTGGAA, R- CACCTGGTTGCTGGC, and UPL probe 29 (Roche) (LightCycler 480; Roche). Renilla luciferase mRNA expression was normalized to that of Renilla luciferase.

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3 SNPs in UCL1 and UCL2, 2 of the 3 SNPs remained significantly associated with bipolar disorder (rs2237563, \( p = .01 \), odds ratio, 1.22; and rs2158766, \( p = .03 \), odds ratio, 0.85) (Table).

### DETECTION AND EVALUATION OF NEW VARIANTS

Eleven SNPs, including a putative Kozak sequence variant (rs148754219), were detected by sequence analysis across the promoter region, 5' UTR, exons, intron/exon junctions, and the 3' UTR of GRM3; an additional 3 SNPs were detected by high-resolution melting analysis of exon 1 and the promoter region (eTable 3). These included synonymous and nonsynonymous coding base pair changes (rs2228595 and rs17161026), a SNP in intron 2 (rs139639092), 5 SNPs in the exon 1 5' UTR (rs184681725, rs2073549, rs48754219, ss518151301, and ss518151302), and 6 promoter SNPs (rs274617, rs274618, rs166677, rs274619, rs274622, and ss518151300). Bioinformatic analysis of the promoter region SNPs for altered transcription factor binding indicated that the mutant alleles of rs148754219, rs166677, and ss518151301 were likely to bind to an increased range of transcription factors compared with their respective common alleles. Prediction of the 5' UTR secondary structure of GRM3 mRNA by the Molpd program showed that the rs148754219 base pair change was not likely to alter the mRNA structure. The variant rs148754219 is located 2 base pairs upstream of the ATG start codon of the GRM3 isofrom GRM3ΔΔ3 (ENST00000546348) encoding ENSP00000441407, thereby becoming a Kozak sequence variant for this isoform.

Ten of these SNPs were selected for genotyping in the complete sample (UCL1 and UCL2) on the basis of an increased frequency in sequenced cases compared with sequenced controls or on predicted functional effects (eTable 3). Of these, the Kozak sequence variant rs148754219 was significantly associated with bipolar disorder (\( p = .005 \), odds ratio, 4.20; 95% CI, 1.43-12.37) (eTable 4) that survived correction for multiple testing (\( p = .047 \)). In the UCL research sample, 19 of the 1099 bipolar cases (1.7%) and only 4 of the 1152 controls (0.3%) were heterozygous for the variant. The association of the rs148754219 variant in the UCL research sample remained significant when only bipolar I cases were included in the analysis (\( p = .006 \)). None of the other new SNPs was associated with bipolar disorder in the complete UCL sample (eTable 4). We combined data from all SNPs genotyped in the GRM3 gene in the present study (UCL1 GWAS SNPs, the 3 GWAS SNPs in UCL2, and 10 SNPs found by mutation screening) and performed haplotype analysis using Haplovie.27 The haplotype block comprising 7 SNPs (rs2299221, rs12704289, rs7804907, rs2237563, rs7806785, rs2299224, and rs17697609) had the strongest association with bipolar disorder that survived permutation testing (\( P = 7.11 \times 10^{-3} \), empirical \( P = .0009 \)). The haplotype driving this association was the same haplotype identified by SNP rs2237563 in the UCL1 GWAS study.2 No other haplotypes were significantly associated with bipolar disorder. The Kozak sequence variant rs148754219 was located outside of this haplotype block but showed linkage disequilibrium with SNP rs2237563 in the haplotype block (\( r^2 = 0.57 \), empirical \( r^2 = 0.001 \)).

### DNA-PROTEIN COMPLEX FORMATION BY RS148754219 VARIANT

The Transcription Element Search System analysis predicted that the rare allele of the rs148754219 variant created transcription factor binding sites recognized by transcription factors, including AP-1, NF-X3, RFX2, E12, and myogenin and the wild-type allele, created binding sites for RAF-1 and GCR1. Electrophoretic mobility shift assays of the variant rs148754219 on transcription factor binding showed that the mutant adenine allele caused gel shifts compared with the wild-type G base sequence. This effect could be abolished by the addition of a 200-fold excess of unlabeled A probe, but not by unlabeled competitor wild-type G probe, indicating that the DNA protein binding was exclusive to the mutant allele (Figure). Similar results were obtained in 3 independent experiments. Supporting the Transcription Element Search System prediction results, the rare A allele of rs148754219 appears to bind more strongly to transcription factors within the nuclear lysate than the wild-type G allele, suggesting that this variant creates a trans...
transcription factor binding site likely to change GRM3 mRNA expression compared with the wild-type sequence.

**EFFECT OF THE RS148754219 VARIANT ON GRM3 PROMOTER ACTIVITY AND ITS FUNCTION AS A TRANSCRIPTION SILENCER**

The ability of the variant rs148754219 to modulate transcription was tested using luciferase reporter gene constructs. Clone A tested the effect of rs148754219 on the ability of GRM3 exon 1 to act as a promoter for luciferase reporter gene activity. We observed a 61% reduction (2-tailed t test, \( P < 10^{-21} \)) in the level of luciferase expression with the mutant clone compared with the wild-type construct in SH-SY5Y cells (eFigure 2C), and no significant difference was observed in HEK293 cells (eFigure 2B). Clone B tested whether similar effects with the rs148754219 alleles could be observed when the GRM3 putative minimal promoter was combined with exon 1. We did not observe any significant differences in expression with the reporter gene driven by the rs148754219 mutant or wild-type alleles in either cell line. Clone C with GRM3 exon 1 cloned upstream of the SV-40 promoter followed by the luciferase gene was used to test whether rs148754219 alleles affected the silencing element present in exon 1. The clone with the mutant base pair A reduced the expression of luciferase by 37% (Mann-Whitney test, \( P < .0001 \)) compared with the wild-type construct in HEK293 cells (eFigure 2B). In contrast, this effect was reversed in SH-SY5Y cells, with an increase in luciferase expression in the mutant construct of 11% (t test, \( P = .04 \)) compared with the wild-type construct (eFigure 2C). Together, these findings indicate that the variant rs148754219 exhibits characteristics of a tissue-specific transcription enhancer element binding site in SH-SY5Y cells that was not active in HEK293 cells.

**ELIMINATION OF LUCIFERASE EXPRESSION DRIVEN BY POSTTRANSCRIPTIONAL/TRANSLATIONAL ACTIVITY OF THE RS148754219 VARIANT**

To investigate whether the rs148754219 variant regulated GRM3 gene expression at the posttranscriptional/translational level rather than at the level of mRNA transcription, we cloned GRM3 exon 1 upstream of the firefly luciferase gene and downstream of the SV-40 promoter. The wild-type and mutant constructs (clone D) were transfected in HEK293 and SH-SY5Y cells. In both cell lines, the clone D mutant rs148754219 led to elimination of firefly luciferase activity (eFigure 2B and C). To elucidate whether the nullifying effect of the mutant allele of rs148754219 on protein expression was related to the in-frame ATG 2 bp downstream of the mutation in the GRM3
5' UTR and not to the ATG of the firefly luciferase gene, we created clone E. In clone E, the A base of the start codon of the luciferase gene was mutated to the base G to give GTG, leaving the ATG immediately downstream of the rs148754219 variant in the GRM3 5' UTR insert as the only in-frame start codon for initiation of translation of firefly luciferase. The rs148754219 mutant clone E construct did not produce any reporter activity in either HEK293 or SH-SY5Y cells, which was similar to the observation for clone D (eFigure 2B and C). These results suggest that the mutant allele A of the rs148754219 polymorphism abolishes downstream post-transcriptional/translational activity. To gain further insight into the molecular effect of the rs148754219 variant on gene regulation, we quantified the firefly luciferase mRNA levels for the wild-type and mutant clone D constructs and compared the results with those of the gene reporter luminescence assays described for the transfection experiments in HEK and SH-SY5Y cells. There was no significant difference in the relative mRNA levels of the wild-type and mutant clones in the HEK293 cell line (wild-type, mean [SE], 0.85 [0.03]; mutant, 0.85 [0.05]; n = 3) and the SH-SY5Y cell line (wild-type, 0.83 [0.01]; mutant, 0.79 [0.03]; n = 3). Therefore, the abundance of firefly luciferase mRNA produced by the clone D construct with the mutant allele suggests that the luciferase gene is being transcribed but not translated. Our results show that the bipolar-associated GRM3 5' UTR rs148754219 variant can be functionally distinguished from the wild-type in different gene reporter assays and that it has an effect at the level of transcription, but the most evident effect is at the posttranscriptional and/or translational level.

The failure to find genetic association in the UCL2 cohort for the markers most strongly associated in the UCL1 sample is typical for the field of complex disease genetics. The difficulty in obtaining clear-cut replications is probably the result of the presence of low-frequency disease alleles and the high degree of etiologic genetic heterogeneity. Significant association was observed for the rs148754219 variant with bipolar I disorder in our sample. The small number of bipolar II cases did not permit a test of association with this variant in our sample. The rs148754219 variant is located in the first exon of GRM3, which is transcribed and not translated in the main isoform of the gene (NM_000840). Bioinformatic analysis predicted that the mutant allele of rs148754219 is bound to several more transcription factors than the wild-type allele. The results from our electrophoretic mobility shift assays provided experimental evidence of this as shown by protein/DNA band shifts with the mutant allele. Based on bioinformatic analysis and the electrophoretic mobility shift assay results, we provide evidence that the rs148754219 variant affects gene expression via the basal transcription apparatus. We also showed that the mutant A allele could cause upregulation of reporter activity in neuroblastoma cells and downregulation in kidney-derived cells. These differences are likely to be the result of variation in the presence of different transcription factors expressed in the 2 cell types.

The luciferase reporter gene assays gave positive results for an effect from the Kozak sequence variant on protein translation. Four alternatively spliced transcripts of GRM3 have been reported in human brain corresponding to full-length GRM3, transcripts with a deletion of exon 2, exons 2 and 3, or exon 4 (eFigure 1). In the GRM3Δ2Δ3 isoform, the SNP rs148754219 is at the −2 position from the translation initiation codon ATG forming part of the Kozak consensus motif, which is a consensus sequence found in eukaryotic mRNA regulating protein translation. We found that the Kozak sequence variant rs148754219 influences the translation efficiency of GRM3Δ2Δ3. Luciferase assays with the mutant 5' UTR cloned between the SV-40 promoter and the firefly luciferase gene showed that the mutant allele caused total suppression of luciferase expression in both cell lines, suggesting that the mutation is harmful. Confirmation that the null reporter activity caused by the mutant allele resulted from the effect on translation initiation from the AUG codon immediately following the rs148754219 variant was found by mutating the luciferase gene start codon. The mRNA levels for the mutant and wild-type alleles were consistent, suggesting that the variant was acting at the posttranscriptional/translational level and not at the level of transcription. Mutations in 5' UTRs affecting translation efficiency have been extensively documented. Kozak sequence SNPs have been reported to be associated with Grave disease, ischemic stroke, Alzheimer disease, and type 2 diabetes mellitus.

The GRM3 gene has been reported to be genetically associated with schizophrenia. Altered levels of dimeric forms of mGluR3 with no change in total mGluR3 have been reported in the prefrontal cortex in patients with schizophrenia. The GRM3 splice isoform GRM3Δ4 encodes a truncated variant of the receptor that has been detected in human brain. This truncated form of the receptor may act as an active competitor for glutamate with the full-length receptor. We have shown that the mutant allele downregulated reporter gene activity, even though mRNA was being transcribed at the same rate as that for the normal wild-type allele. This can be explained by ribosomal scanning, which is involved in the internal ribosomal entry site–mediated, cap-independent, translational control of mRNA. During the scanning process, the Kozak sequence variant may cause the ribosomes to ignore the AUG of the GRM3Δ2Δ3 transcript, a process called “leaky-scanning,” and recognize other downstream translational start sites, such as the AUG codon of full-length GRM3, GRM3Δ2, and/or GRM3Δ4. The knowledge of the expression of the alternatively spliced transcripts of GRM3 and their respective protein products in postmortem brains from individuals with bipolar disorder may provide more insight into the etiology of bipolar disorder.

Successful effects of class II mGluR3 drugs in treating anxiety and stress-related disorders have been reported. Expression data from animal models of depression suggest that mGluR2/3 mRNA expression is reduced in the hippocampus. Increased levels of the mGluR2/3 proteins also have been observed in the pre-
frontal cortex of patients with major depressive disorder, whereas both elevated41 and reduced expression42,43 levels of mGluR2/3 in the frontal cortex of patients with schizophrenia have been observed. Several mGluR2/3 agonists and antagonists have been established. Clinical studies on mGluR2/3 agonist LY354740 in the treatment of anxiety10,11 and its LY404309 prodruk LY2140023 in the treatment of schizophrenia12-14 have been conducted with positive outcomes. MGS0039 is a selective mGluR2/3 agonist that has antidepressant-like activity in rat models.34 The rs148754219 variant has not been reported in any sample other than the 1000 Genomes Database samples (http://www.1000genomes.org). In our sample, the Kozak sequence variant was convincingly overrepresented in bipolar disorder cases compared with controls, with an odds ratio above 4, suggestive of moderate risk for bipolar disorder. To our knowledge, this is the first study that reports a possible, although unconfirmed, rare and functional base pair change that is significantly associated with bipolar disorder. The finding needs confirmation in additional samples of unipolar and bipolar affective disorder as well as in alcoholism, where unipolar affective disorder is known to be a primary etiologic factor.

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