Neuropsychopharmacology (2008) 33, 2626–2634

www.neuropsychopharmacology.org

Expression of a GRM3 Splice Variant is Increased in the Dorsolateral Prefrontal Cortex of Individuals Carrying a Schizophrenia Risk SNP

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Genetic variation in the metabotropic glutamate receptor 3 (GRM3, mGluR3) has been associated with schizophrenia, but the mechanism by which it confers risk is unknown. Previously, we reported the existence of a splice variant, GRM3Δ4, which has an exon 4 deletion and encodes a truncated form of the receptor that is expressed in brain. The aim of the present study was to determine whether expression of this splice variant is altered in individuals with schizophrenia and is affected by a risk genotype. We measured GRM3 and GRM3Δ4 transcripts in human dorsolateral prefrontal cortex (DLPFC) and hippocampus of the CBDB/NIMH collection (~70 controls, ~30 schizophrenia patients) and in the DLPFC of the Stanley Array Collection. Expression data of GRM3 mRNA in the DLPFC were inconsistent: GRM3 was increased in schizophrenia patients in the CBDB/NIMH collection, but not in the Stanley Array Collection. GRM3 expression did not change in the frontal cortex of rats treated chronically with haloperidol or clozapine. An exon 3 SNP previously associated with schizophrenia (rs2228595) predicted increased expression of the GRM3Δ4 splice variant. Our results suggest that rs2228595, or a neighboring SNP in linkage disequilibrium with it, may contribute to risk for schizophrenia by modulating GRM3 splicing.

Neuropsychopharmacology (2008) **33,** 2626–2634; doi:10.1038/sj.npp.1301669; published online 6 February 2008

Keywords: post mortem; metabotropic; glutamate; mGluR3; neuroleptic; bipolar disorder

INTRODUCTION

Metabotropic glutamate receptors (GRMs; mGluRs) contribute to the regulation of synaptic glutamate. These receptors are subdivided into three groups (I–III) based on sequence homology, signal transduction, and pharmacological properties (Conn and Pin, 1997). Group II comprises two receptors, GRM2 and GRM3, which have high sequence homology and are difficult to distinguish with specific ligands and antibodies. Since GRM2/3 activation leads to a net reduction in glutamate neurotransmission (Battaglia *et al*, 1998; East *et al*, 1995; Lovinger and McCool, 1995; Yoshino *et al*, 1996), ligands for these receptors are considered as potential therapeutic agents for a wide range of psychiatric disorders (Javitt, 2004). For example, GRM2/3 agonists reverse cognitive and motor deficits in a rodent NMDA receptor antagonism model for schizophrenia (Moghaddam and Adams, 1998; Olszewski *et al*, 2004). Mechanistically, it has been proposed that GRM2/3 agonists reverse the cortical glutamate efflux that is induced by NMDA antagonist administration, and which has been linked to the cognitive dysfunction. Furthermore, there is preliminary evidence in humans that group II agonists are effective in improving cognitive deficits induced by ketamine (Krystal *et al*, 2005) and that they possess antipsychotic activity (Patil *et al*, 2007).

In addition to a potential role for GRM2/3 agonists in treating schizophrenia, GRM3 is a candidate gene for this disorder. SNPs or haplotypes in GRM3 have been associated with schizophrenia in several independent samples (Chen *et al*, 2005; Egan *et al*, 2004; Fujii *et al*, 2003; Marti *et al*, 2002; Schwab *et al*, 2006), although negative studies have been reported as well (Marti *et al*, 2002; Norton *et al*, 2005; Tochigi *et al*, 2006). Recently, polymorphisms in GRM3 have also been associated with bipolar disorder (Fallin *et al*, 2005; Green *et al*, 2006), which shares points of genetic vulnerability with schizophrenia. Finally, SNPs in GRM3 are associated with cognitive performance in normal control subjects (de Quervain and Papassotiropoulos, 2006; Egan *et al*, 2004). However, the mechanisms underlying these genetic associations are unclear, since all known common

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Received 5 November 2007; revised 17 December 2007; accepted 17 December 2007

Table 1 Characteristics of Subjects Used for mRNA Expression Measurements

	n	Race	Sex	age	pH	PMI	RIN
NIMH prefrontal cortex							
Control	70	22C, 45AA, 3H	50M, 20F	40.4 (15.7)	6.6 (0.22)	32.3 (15.5)	6.8 (1.4)
Schizophrenic	34	15C, 17AA, 2H	20M, 14F	48.24 (18.4)	6.5 (0.23)	36.2 (14.9)	6.5 (1.7)
NIMH hippocampus							
Control	71	23C, 44AA, 4H	55M, 16F	40 (15.1)	6.6 (0.26)	30.7 (13.7)	5.7 (1.2)
Schizophrenic	30	14C, 14AA, 2H	18M, 12F	47.2 (18.7)	6.5 (0.26)	38.8 (16.30)	5.3 (1.1)
Stanley prefrontal cortex							
Control	35	35C	26M, 9F	44.2 (29.30)	6.6 (0.26)	29.4 (12.9)	n/a
Schizophrenic	33	32C, 1H	25M, 8F	42.7 (8.7)	6.4 (0.24)	30.8 (15.6)	n/a
Bipolar disorder	33	32C, 1AA	15M, 18F	46.2 (9.7)	6.4 (0.30)	37.7 (17.4)	n/a

Values are listed as mean (SD). C, Caucasian; AA, African American; H, Hispanic; M, male; F, female; PMI, post-mortem interval (h); RIN, Agilent RNA integrity number (on a scale of 1–10).

SNPs within GRM3 are either noncoding or synonymous, and thus do not alter the amino-acid sequence of the translated receptor. It is possible that rare, still-undiscovered coding SNPs exist, but it is more probable that the noncoding SNPs themselves, or other SNPs with which they are in linkage disequilibrium (LD), are functionally associated with the disorder.

One way that noncoding SNPs in GRM3 may confer risk for schizophrenia (or other phenotypes) is by impacting the expression levels of full-length GRM3 or alternative transcripts. Recently, we discovered an alternatively spliced form of GRM3, called GRM3Δ4, which lacks the transmembrane domain exon 4 and encodes a truncated variant of the receptor (Sartorius *et al*, 2006). The variant is present in the brain of both schizophrenia patients and controls, and thus its presence or absence is not sufficient to explain the gene's contribution to the disorder. However, it is plausible that relative quantities of GRM3 transcripts are altered in individuals with schizophrenia or with a risk genotype. We were particularly interested in studying SNPs located near the spliced fourth exon, which may be in a position to interact with the splicing machinery. To this end, we quantified mRNA levels of GRM3 and GRM3Δ4 in the dorsolateral prefrontal cortex (DLPFC) and hippocampus from a collection of post-mortem brains of controls and schizophrenia patients ('CBDB/NIMH collection'). We repeated the DLPFC study in a second cohort, the Stanley Array Collection. We also studied the frontal cortex of rats chronically treated with clozapine or haloperidol to determine whether GRM3 expression in the patients with schizophrenia may have been affected by medication.

MATERIALS AND METHODS

Human Post-mortem Tissue

From the CBDB/NIMH collection, described in detail elsewhere (Lipska *et al*, 2006a), we used tissue homogenates from the DLPFC gray matter ($n=70$ controls, $n=34$ schizophrenia patients) and hippocampus ($n=71$ controls, $n=30$ schizophrenia patients), taken from largely overlapping subjects ($\sim$95%; Table 1). For the DLPFC, gray matter tissue was obtained from coronal slabs corresponding to the middle one-third of the middle frontal gyrus, immediately anterior to the genu of the corpus callosum. White matter was carefully trimmed off using a dental drill. The hippocampal formation was identified on 2–3 frozen coronal slabs from the medial temporal lobe and dissected using a dental drill. The lateral ventricle and the fimbria-fornix were used as the medio-dorsal boundary and the subiculum and underlying white matter as the ventral boundary. The adjacent para-hippocampal cortex was not included in the dissection.

Brain tissue in the CBDB/NIMH collection was obtained with informed consent from the legal next of kin under NIMH protocol no. 90-M-0142. Diagnoses were made by independent reviews of clinical records by two board-certified psychiatrists using DSM-IV criteria. Subjects were designated as normal controls based on a standardized screening interview with next of kin, in addition to a review of all available medical records and interviews conducted by the investigators at the medical examiners' offices. Brain tissue was screened by a neuropathologist, and all subjects with neuropathological abnormalities were excluded. Toxicological analysis was conducted for every case using blood and/or brain specimens for both drugs of abuse and psychotherapeutic medication. Nonpsychiatric cases with toxicology screenings positive for ethanol above the American Medical Laboratory Inc. limit (0.05 g/dl), positive for any medication above therapeutic levels, or positive for any illicit drugs were excluded from the control group. Positive toxicology was not an exclusion criterion for cases with schizophrenia.

The Stanley Array Collection, an independent cohort obtained from the Stanley Medical Research Institute, consists of 105 post-mortem brains of schizophrenia patients, bipolar disorder patients, and control individuals (Stanley Array Collection, described at http://www.stanleyresearch.org/programs/brain_collection.asp). Four cases were missing in our analysis due to insufficient sample quantities, leaving

33 bipolar disorder patients, 33 schizophrenia patients, and 35 controls (Table 1). These specimens were collected, with informed consent from next of kin, by participating medical examiners between January 1995 and June 2002. Exclusion criteria included structural brain pathology, history of significant focal neurological signs premortem, documented IQ <70, and poor RNA quality. Additional exclusion criteria for controls included age <30 (thus, still in the period of maximum risk), or substance abuse within 1 year of death or evidence of significant alcohol-related changes in the liver. The specimens were all collected, processed, and stored in a standardized way. Total RNA was extracted from the homogenate of mostly gray matter of the DLPFC and processed as described below.

Neuroleptic Treatment in Rats

Male Sprague–Dawley rats (weight ~ 250 g) were on a 12-h light/dark cycle (lights on/off 0600 hours/1800 hours) in a temperature-controlled environment and with *ad lib* access to food and water. Rats were randomly assigned to drug treatment groups (8–10 per dose) and administered intraperitoneal injections of haloperidol (0.08, 0.6, and 2 mg/kg), clozapine (0.5, 5, and 10 mg/kg), or vehicle (0.02% lactic acid) once daily for 28 days. Haloperidol (Research Biochemicals Inc., Natick, MA, USA) (20 mg/ml) was prepared in 1% lactic acid, diluted with water, and neutralized with 1 M NaOH to obtain pH 5.3. Clozapine (a gift from Sandoz Research Institute Berne Ltd, Berne, Switzerland) (100 mg) was dissolved in 0.1 M HCl, diluted with water, and neutralized to pH 5.2. Rats were killed 7 h after the last injection. Frontal cortex without white matter was dissected and frozen at -80°C. All procedures were performed in accordance with the National Institutes of Health Guidelines for Use and Care of Laboratory Animals.

RNA Extraction and Reverse Transcription

Brain tissue was pulverized and stored at -80°C. Total RNA was isolated from 100 mg of brain tissue using TRIZOL Reagent (Life Technologies Inc., Grand Island, NY, USA) according to the manufacturer's protocol. The yield of total RNA was determined by absorbance at 260 nm. RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and samples showing clearly defined, sharp 18S and 28S ribosomal peaks, 28S/18S ratios >1.2, and RNA integrity number (RIN) >4.0 for the DLPFC and >3.8 for the hippocampus were included. Although RINs were not measured in the Stanley Array Collection due to limited sample quantities, the RNA quality of these samples based on the Agilent 28S/18S ratios was comparable to the CBDB/NIMH collection. Agilent traces did not show anomalous peaks indicating genomic DNA contamination in any sample. First-strand cDNA was generated from 5 μg of total RNA using random hexamers in conjunction with 125 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a final reaction volume of 50 μl. The cDNA was treated with 10 U of ribonuclease H (Invitrogen) to remove bound RNA template.

Quantitative Real-Time PCR

Expression levels of mRNAs were measured by real-time quantitative PCR using the ABI Prism 7900 sequence detection system, 384-well format (Applied Biosystems, Foster City, CA, USA). Each 20 μl reaction contained cDNA template obtained from 200 ng RNA, 900 nM primer, 250 nM probe, and 1 × Taqman Universal PCR Mastermix (Applied Biosystems) with Hot Goldstar DNA Polymerase, dNTPs with dUTP, uracil-*N*-glycosylase.

To measure full-length mRNA, all isoforms, and a variant with exon 4 deletion, we used Applied Biosystems Assay-on-Demand (GRM3_full, Hs 00168260, spanning exons 3–4) and two sets of custom-designed primers and probes: GRM_Pan probe was designed to amplify a region of exon 6 and thereby to detect all known GRM3 isoforms (for: ACACACAGACTGCACCTCAACAG; rev: TGCACACCGTTG GCACAT; probe: CTCTCAGTCCTCTGCAAG) and GRM3Δ4 probe was designed to span the exon 3–5 junction (for: ATGAAGATCCTGGATGGGAAGAA; rev: CAAAGCCACTC AGGCTGACA; probe: ATCAACTTCACGGGTGCA). For normalization, we used two housekeeping genes porphobilinogen deaminase (Hs00609297_m1) and β2-microglobulin (Hs99999907). For rats, we used assays for GRM3 (Rn01755349_m1), B2M (Rn00560865_m1), and PGBD (Rn00565886_m1).

PCR cycle parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 59 or 60°C for 1 min. PCR data were acquired from the Sequence Detector Software (SDS version 2.0, Applied Biosystems) and quantified by a standard curve method using serial dilutions of pooled cDNA derived from RNA obtained from the corresponding region of 10–12 normal control subjects. In each experiment, the R^2 value of the curve was more than 0.99, the slope was between -3.2 and -3.5 (amplification efficiency 93–105%), and controls that lacked template cDNA emitted no detectable signal. All samples were measured in a single plate for each gene, and their cycles at threshold (C_t) values were in the linear range of the standard curve. All measurements were performed in triplicate.

GRM3 Genotype and Diplotype Determination

We analyzed SNPs that had been previously associated with schizophrenia (rs6465084, rs2228595, and rs1468412), an intron 4 SNP (rs7804100) that is part of the high-risk haplotype in our prior study (Egan *et al*, 2004), and four additional SNPs that were selected from HapMap as tag SNPs for haplotypes in the 5′ regulatory region of the gene (rs1527768, rs906415, rs802457, and rs187993; Table 2). DNA was extracted from cerebellar tissue using a protocol by PUREGENE (Gentra Systems, Minneapolis, MN, USA). Genotyping was performed using the Taqman 5′-exonuclease allelic discrimination assay, as described elsewhere (Egan *et al*, 2004). Reproducibility was routinely assessed by re-genotyping all samples for selected SNPs and was generally $>99\%$. The program SNPHAP written by David Clayton (version 1.0) was used to calculate frequencies of two four-marker haplotypes (SNPs 1–4 and SNPs 5–8) and to assign diplotypes to individuals.

Statistical Analyses

All analyses were conducted blind to diagnosis. Statistical analyses were performed using SPSS 12.0.1 (Apache Software Foundation). Comparisons between diagnostic and genotypic groups were made using ANCOVA with diagnosis or genotype as the independent variable and demographic/tissue-related variables as covariates. Covariates were those variables that were significantly correlated with the abundance of a particular transcript as measured by Spearman's rank order correlation, from among factors including age, pH, PMI, RIN, duration of illness, and daily, lifetime, and last neuroleptic dosages. When appropriate, *post hoc* analysis was performed using a Fisher's least significant difference (LSD) test. The geometric mean of the housekeeping genes was used as a normalizing factor (Lipska *et al*, 2006b; Vandesompele *et al*, 2002). The GRM3Δ4 transcript was also normalized to GRM3_Pan and GRM3_full. In rats, only GRM3 was measured (since we have been unable to detect unequivocally the presence of GRM3Δ4). In the entire sample, extreme outliers (defined as normalized measures that lay further than (3 × interquartile range) from the median) for each expression variable were eliminated from further analysis ($n = 1$–3). When the sample sizes were unbalanced and/or variances unequal, we used a nonparametric test (Mann–Whitney *U*-test). To increase power in the genotype analysis, we also combined the CBDB/NIMH and Stanley DLPFC qPCR results following *Z*-score transformation of the data sets ($Z =$ (data point value−group mean)/group standard deviation). For clarity, the data in bar graphs are presented as 'percent of controls.'

Table 2 Summary of SNPs Analyzed

SNP	dbSNP rs no.	Location	SCZ assoc.
1	rs1527768	Promoter	
2	rs906415	Promoter	
3	rs802457	Promoter	
4	rs187993	Promoter	
5	rs6465084	Intron 2	Egan *et al* (2004)
6	rs2228595	Exon 3	Marti *et al* (2002)
7	rs1468412	Intron 3	Fujii *et al* (2003)
8	rs7804100	Intron 4	

RESULTS

Effect of Diagnosis on Expression of GRM3 Isoforms

In the CBDB/NIMH collection, GRM3_Pan transcripts and GRM3_full transcript were modestly (by ~9 and 8%, respectively) but significantly ($F(2, 94) = 6.68$; $p = 0.01$, Figure 1a, and $F(2, 94) = 5.0$; $p = 0.02$, data not shown) increased in the DLPFC of schizophrenia patients. We did not find a significant effect of diagnosis on any of the GRM3 transcripts' expression in the CBDB/NIMH hippocampus (all $F(2, 90) < 1.5$, $p > 0.2$; Figure 1b). In the Stanley DLPFC, there was a trend for diagnosis to affect GRM3_Pan expression ($F(3, 95) = 2.4$, $p = 0.1$), related to increased levels in bipolar disorder patients (Figure 1c). Finally, we did not observe a diagnosis effect on GRM3Δ4 mRNA expression in any cohort (all $F < 1.60$, all $p > 0.20$; data not shown). We used age as a co-variate in these analyses, as age correlated inversely with the expression of GRM3_Pan, GRM3_full, and GRM3Δ4 mRNA (Spearman's *R*-values ranging from −0.36 to −0.25, $p < 0.001$). Other factors (RIN, pH, PMI) did not correlate with the expression of GRM3 transcripts.

Effect of Neuroleptics on GRM3 Expression

Neuroleptic medications can impact mRNA levels in the post-mortem brain, possibly accounting for the slight increase in GRM3 expression in the DLPFC of the CBDB/NIMH collection. There was, however, no significant

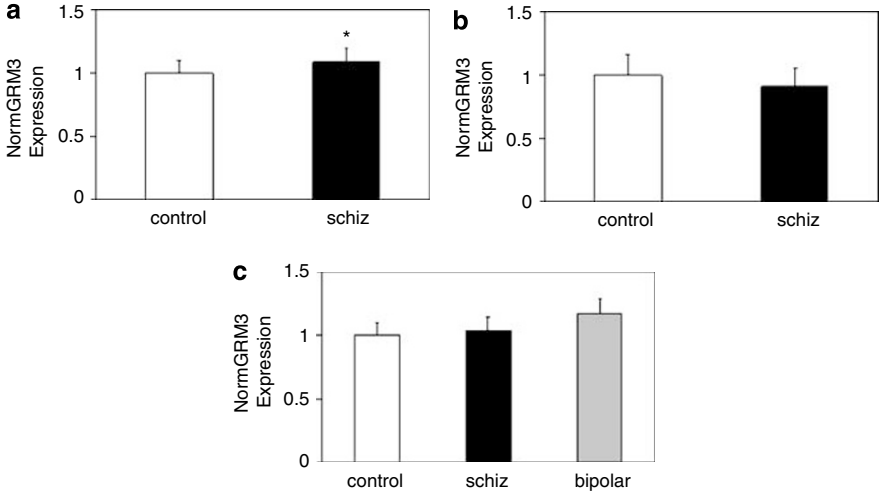

Figure 1 Expression of GRM3 mRNA in schizophrenic patients and control individuals. Quantitative RT-PCR analysis of normalized GRM3_Pan mRNA (NormGRM3) expression. (a) CBDB/NIMH DLPFC, $n = 69$ controls and 31 schizophrenics. (b) CBDB/NIMH hippocampus, $n = 70$ controls and 28 schizophrenics. (c) Stanley DLPFC (includes bipolar disorder patients), $n = 33$ controls, 33 schizophrenics and 33 bipolar disorder patients. Bars outside the box represent the standard deviation from the mean. *Significant difference ($p < 0.01$).

correlation between CPZ equivalents (daily, lifetime, and last neuroleptic doses) and expression of any of the GRM3 isoforms in any brain region (Spearman's R-values <0.2, p-values >0.2; data not shown). We also measured GRM3 mRNA expression in the frontal cortex of rats treated with three doses of haloperidol (0.08, 0.6, and 2.0 mg/kg) and clozapine (0.5, 5.0, and 10 mg/kg), and of drug-naïve rats. We found no significant difference in GRM3 expression between the treatment and control groups ($F(6, 52) = 0.92$, $p = 0.92$; Figure 2).

Other potentially confounding factors, that is, smoking and substance abuse, also did not have any effect on GRM3 or GRM3Δ4 expression in any cohort ($p > 0.5$, data not shown).

Effect of GRM3 Genotype on GRM3 Expression

The infrequent 'T' allele of SNP 6 (exon 3 Ala293Ala; rs2228595) was previously associated with risk for schizophrenia (Egan *et al*, 2004; Marti *et al*, 2002). In the CBDB/NIMH DLPFC collection, individuals carrying the 'T' allele ($n = 9$, all heterozygotes) had ~21% higher expression of GRM3Δ4 compared to C/C individuals ($n = 80$) (Mann–Whitney U-test, $Z = -2.58$, $p = 0.008$, ANCOVA: $F = 5.67$, $p = 0.008$; Figure 3a). In the hippocampus, GRM3Δ4 expression was also higher in C/T individuals, but this difference did not reach statistical significance ($Z = -1.44$, $n = 83$, $p = 0.15$, ANCOVA: $F = 3.01$, $p = 0.1$; Figure 3b). In the Stanley collection, T carriers ($n = 6$) had ~8% higher expression of GRMΔ4 compared to C/C individuals, but this difference was not significant (Figure 4c; $Z = -0.79$, $n = 98$, $p = 0.43$, ANCOVA: $F = 1.9$, $p = 0.38$; Figure 3c). Given the small sample size for T carriers in each of these groups, we combined the CBDB/NIMH and Stanley DLPFC data. In the combined data set, T carriers showed significantly increased expression of GRM3Δ4 (Figure 4d; $Z = -2.53$, $n = 187$, $p = 0.011$; Figure 3d).

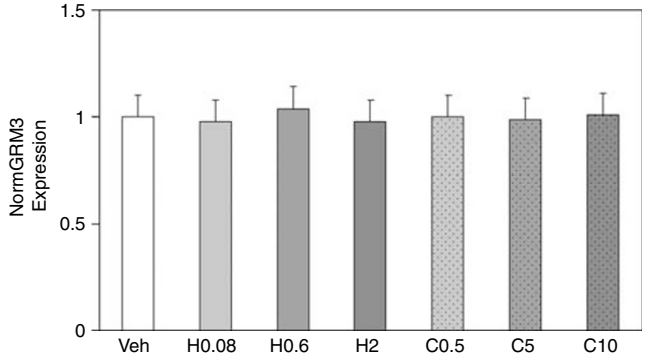

Figure 2 Expression of prefrontal normalized GRM3 mRNA (NormGRM3) in neuroleptic-treated rats. Bars outside the box represent the standard deviation from the mean. VEH, vehicle; H0.08, haloperidol 0.08 mg/kg; H0.6, haloperidol 0.6 mg/kg; H2, haloperidol 2 mg/kg; C0.5, clozapine 0.5 mg/kg; C5, clozapine 5 mg/kg; C10, clozapine 10 mg/kg.

SNP 8, located in intron 4, also showed association with GRM3Δ4 expression in the CBDB/NIMH DLPFC ($F(3, 86) = 10.7$, $p < 0.001$; Figure 4a). *Post hoc* testing revealed that A/G heterozygous individuals had lower expression levels than A/A ($p = 0.001$) and G/G ($p < 0.001$) homozygous individuals. This association was also significant in the CBDB/NIMH hippocampus ($F(2, 81) = 6.05$, $p = 0.004$; Figure 4b). Again, expression in A/G individuals was significantly lower than in A/A individuals (LSD: $p = 0.001$) and marginally lower than in G/G individuals ($p = 0.058$). In the Stanley DLPFC collection, genotype at SNP 8 was not associated with levels of normalized GRM3Δ4 ($F(2, 95) = 0.13$, $p = 0.88$; Figure 4c). In the combined CBDB/NIMH and Stanley DLPFC data set, SNP 8 did affect global GRM3Δ4 levels ($F(3, 184) = 5.2$, $p = 0.006$), with levels in heterozygotes being significantly lower than in A/A ($p = 0.028$) or G/G homozygotes ($p = 0.004$; Figure 4d).

None of the eight SNPs analyzed, including the promoter SNPs, predicted changes in GRM3_Pan or GRM3_full expression in CBDB/NIMH DLPFC (all F-values <1.6, all

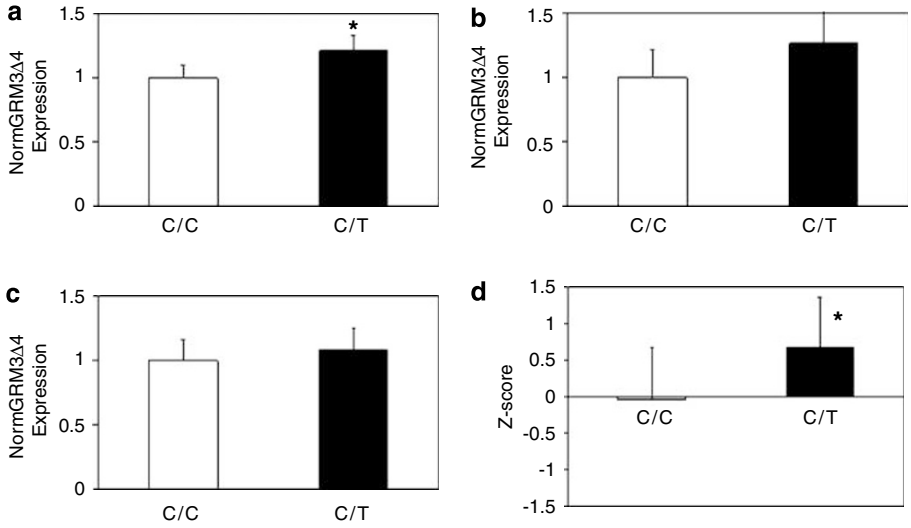

Figure 3 Association between SNP rs2228595 and GRM3Δ4 transcript expression. Quantitative RT-PCR analysis of normalized GRM3Δ4 mRNA expression (NormGRM3Δ4). (a) CBDB/NIMH DLPFC, $n = 80$ C/C and 9 C/T carriers. (b) CBDB/NIMH hippocampus, $n = 77$ C/C and 6 C/T carriers. (c) Stanley DLPFC, $n = 92$ C/C and 6 C/T carriers. (d) Combined NIMH and Stanley DLPFC (Z-score), $n = 172$ C/C and 15 C/T carriers. Bars outside the box represent the standard deviation from the mean. *Significant difference ($p < 0.05$).

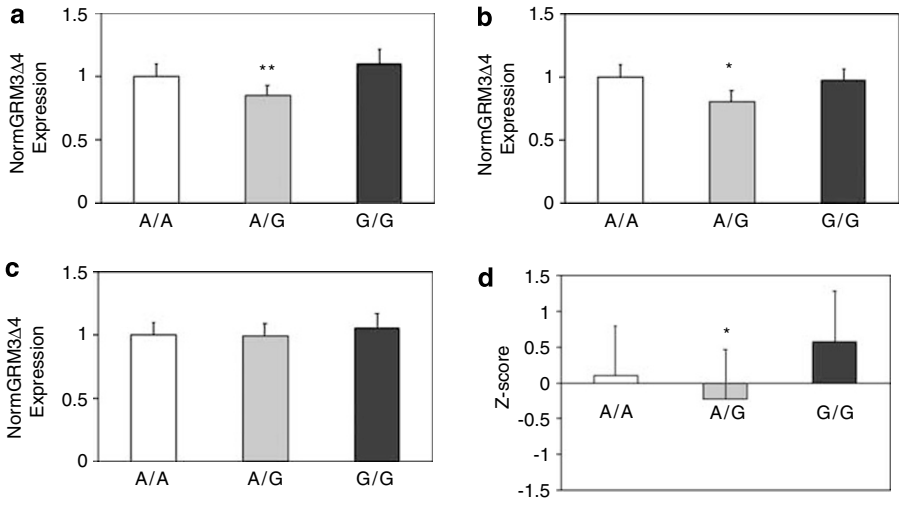

Figure 4 Effect of SNP 8 on GRM3Δ4 transcript expression. Quantitative RT-PCR analysis of normalized GRM3Δ4 (NormGRM3Δ4). (a) CBDB/NIMIH DLPFC, $n = 40$ A/A, 39 A/G, and 11 G/G carriers. (b) CBDB/NIMH hippocampus, $n = 36$ A/A, 37 A/G, and 11 G/G carriers. (c) Stanley DLPFC, $n = 51$ A/A, 44 A/G, and 3 G/G carriers. (d) Combined NIMH/Stanley DLPFC (*Z*-score), $n = 91$ A/A, 83 A/G, and 14 G/G carriers. Bars outside the box represent the standard deviation from the mean. *Significant difference ($p < 0.01$), **($p < 0.001$).

$p > 0.2$), CBDB/NIMH hippocampus (all F-values < 2.0, all $p > 0.2$), or Stanley DLPFC (all F-values < 1.22, all $p > 0.27$) (data not shown).

DISCUSSION

In the present study, we examined the effects of schizophrenia diagnosis and risk genotypes on the expression of GRM3 mRNA isoforms. We previously showed that GRM3 has an abundant splice variant lacking exon 4, which encodes a truncated protein in the human brain predicted to be missing the critical transmembrane portion of the protein (Sartorius *et al*, 2006). Although the function of this novel and putatively soluble protein is unknown, we hypothesized that it may interact with the full-length receptor via heterodimerization, or it may act as a 'decoy receptor,' effectively sequestering glutamate and acting in a dominant-negative manner with the full-length receptor.

Effect of Diagnosis on Expression of GRM3 Transcripts

In the present study, we found a slight increase in DLPFC GRM3 mRNA expression in schizophrenia in the CBDB/NIMH collection, consistent with one prior study of GRM3 protein (Gupta *et al*, 2005). However, we did not replicate the finding in the Stanley Array Collection, and other studies of the DLPFC GRM3 mRNA (Ohnuma *et al*, 1998) and GRM3 immunoreactivity (Crook *et al*, 2002) have been negative as was the study in the thalamus (Richardson-Burns *et al*, 2000). We also found GRM3 unchanged in the hippocampus. Thus, given that our study is by far the largest to date, we conclude that there is no convincing evidence for altered GRM3 expression—or splicing—in schizophrenia. However, it is still possible that such alterations occur in a genetically predisposed subset of affected individuals, or that protein levels are altered in the absence of consistent changes in mRNA or that GRM3 receptors are altered in other ways. For example, Corti *et al* (2007) found a marked decrease in dimeric forms of GRM3 in the prefrontal cortex in schizophrenia, with no change in total GRM3. It is also possible that changes are region or layer specific. Further studies are therefore warranted to investigate the possibility of subtle, yet potentially functionally significant, alterations in GRM3 expression in schizophrenia.

We found no correlation between neuroleptic treatment and GRM3 or GRM3Δ4 expression in human samples and no effect of haloperidol or clozapine on GRM3 mRNA in rats. To date, the only other study to address this question showed an increase in GRM3 mRNA expression in the rat frontal cortex after chronic exposure to clozapine and, to a lesser extent, olanzapine, but not haloperidol (Tascedda *et al*, 2001). The reason for the inconsistency with our rat data is uncertain. Taken together, our data suggest that neuroleptic drugs are unlikely to alter GRM3 mRNA expression in schizophrenia.

Effects of SNPs on GRM3 Transcript Expression

We selected eight SNPs to test for association with altered expression or splicing of the GRM3 mRNA transcript. Four of them were selected as tag SNPs for haplotypes in the 5′ regulatory region of the first exon of GRM3, and we predicted that these may impact expression of GRM3. The remaining four SNPs (which had been previously associated with schizophrenia, either individually or as part of a risk haplotype) were located around exons 3 and 4, and we predicted that these might regulate alternative splicing of the fourth exon. In keeping with this prediction, two of the SNPs were associated with expression of the GRM3Δ4 splice variant. Overall, GRM3 mRNA levels were not affected by any of the SNPs.

In our analysis of GRM3Δ4 transcript expression, we were particularly interested in rs2228595 (SNP 6; exon 3 Ala/Ala). In addition to being the first polymorphism associated with schizophrenia (Egan *et al*, 2004; Marti *et al*, 2002), this is the only common exonic SNP known so far to exist in GRM3.

Furthermore, this synonymous SNP is located in exon 3, relatively close to the alternatively spliced fourth exon. Although it does not map to a known exonic splicing enhancer or inhibitor site (Cartegni *et al*, 2003; Fairbrother *et al*, 2002), we hypothesized that the SNP might nonetheless impact splicing in a way that is not predicted *in silico*, or it may be in LD with other undiscovered SNPs involved with the splicing machinery. We observed that in the CBDB/NIMH DLPFC collection, presence of the minor frequency 'T' allele predicted ~20% higher relative expression of GRM3Δ4. If indeed this SNP predicts increased levels of truncated protein, which may retain the ability to dimerize with the full-length GRM3 receptor, this may affect dimerization and function of GRM3 in schizophrenia, as suggested by a recent study (Corti *et al*, 2007). We attempted to replicate this in the Stanley brain collection, but the minor allele was of even lower frequency in that collection, and the result was equivocal. However, when we used statistical normalization to combine the data from both collections (as the experiments were carried out separately, and the raw data could not be combined), the finding remained robust.

Although we have shown that the minor frequency 'T' allele at SNP 6 predicted ~20% higher relative expression of GRM3Δ4 in all subjects, we did not see a significant increase in the level of this transcript in schizophrenia. This is not surprising given that the effect size is relatively small, the frequency of this allele in this population is low, and the enrichment of the allele in schizophrenia is small. Thus, we had insufficient power to detect the change in patients in this study. This, however, does not preclude the possibility that this SNP contributes to risk for schizophrenia, albeit to a small degree and perhaps in conjunction with other SNPs/genes of relatively small effects. Moreover, allelic variation in multiple genes contributing small effects is probably the norm for complex genetic disorders such as schizophrenia.

A second SNP that predicted levels of the GRM3Δ4 splice variant was SNP 8, located in the middle of the 10 kb fourth intron. In both the CBDB/NIMH and the Stanley DLPFC collections, heterozygotes appeared to have lower levels of the GRM3Δ4 splice variant than either A/A or G/G homozygotes. This finding might be explained by the phenomenon of 'molecular heterosis,' wherein subjects heterozygous for a polymorphism demonstrate a significantly greater (positive heterosis) or lesser (negative heterosis) effect for a quantitative trait than homozygous subjects (Comings and MacMurray, 2000). However, it would be difficult to substantiate the hypothesis that splicing is altered by heterozygosity at an intronic SNP. As an alternative, we explored the possibility that the decreased GRM3Δ4 expression in A/G individuals is an epiphenomenon of genetic background at the other SNP that affected expression (SNP 6). Analyzing the common haplotypes in the CBDB/NIMH collection, we found that heterozygosity at SNP 8 was almost always on the background of the common 'C' allele for SNP 6. Since the SNP 6 'C' allele is associated with relatively lower expression of GRM3Δ4, the SNP 8 heterozygotes will also have low expression compared to homozygotes.

It is possible that none of the SNPs associated with GRM3Δ4 expression are functional, but instead tag nearby functional SNPs. To address this, we constructed haplotypes to determine whether the signal was stronger when combined with other SNPs in the region. We also assigned diplotypes, which describe the likely haplotype status on each chromosome. We did not observe an association between haplotype or diplotype status and GRM3 isoform expression in any sample perhaps, because there were very few individuals in each haplotype/diplotype group and our SNP coverage of this region of the gene is limited.

We did not perform corrections for multiple testing in our analysis. Such correction would be unduly conservative given that we restricted our analysis to planned comparisons (selecting SNPs that were previously associated with schizophrenia, or were in the 5′ regulatory region of the gene). Furthermore, several of the SNPs are in moderate LD and thus their degree of independence is low, making correction for multiple testing inappropriate. Nonetheless, our finding regarding an association of GRM3 splicing with SNP rs2228595 requires independent replication.

Concluding Remarks

GRM3 is one of over a dozen genes that have been putatively identified as being associated with increased risk for schizophrenia (Harrison and Weinberger, 2005; Straub and Weinberger, 2006). A major challenge in validating the candidacy of schizophrenia genes is thus to determine how these SNPs impact the molecular phenotype of the cell, leading to downstream alterations in brain function. As is the case with GRM3, most of the SNPs that have been associated with schizophrenia are noncoding, and therefore do not translate into an easily identifiable functional output.

One way that noncoding SNPs can impact cell function is by altering the expression of mRNA transcripts, including splice variants. For example, in a recent study of neuregulin-1, another schizophrenia susceptibility candidate gene, an SNP in the 5′ region of the gene predicted specifically increased expression of the novel type IV transcript, which arises from alternative 5′ exon usage (Law *et al*, 2006).

In the present study, we found that an exon 3 SNP in GRM3 predicts increased splicing of the fourth exon. This finding is relevant to basic GRM3 biology, since it may alter the translation profile of the novel protein encoded by GRM3Δ4, and in turn impact GRM3 function. Further, since this SNP has been previously associated with schizophrenia, it raises the possibility that altered splicing of GRM3 is one mechanism by which the gene contributes to the disorder.

ACKNOWLEDGEMENTS

LJS is a student in the NIH/Oxford Graduate Partnership Program. This research was supported by the Intramural Research Program of the NIH, NIMH. Post-mortem brain tissue was donated by the Stanley Medical Research Institute, courtesy of Drs Michael B Knable, E Fuller Torrey, Maree J Webster, Serge Weis, and Robert H Yolken. We thank Ms Cara Horowitz, Ms Tricia Peters, and Mr Krishna Vakkalanka for their excellent technical assistance, Dr Mary M Herman for her neuropathological assessments, and Amy Deep-Soboslay for her work on the demographic and clinical data. We thank the staff of the Offices of the Chief

Medical Examiner of District of Columbia and of Northern Virginia for their assistance. We also thank the families of the deceased, who generously donated brain tissue as well as their time and effort to make this study possible.

DISCLOSURE/CONFLICT OF INTEREST

Dr Harrison's research is supported by the United Kingdom Medical Research Council, Stanley Medical Research Institute, National Alliance for Research on Schizophrenia and Depression, Wellcome Trust, and an unrestricted grant from GlaxoSmithKline. In the past 3 years, Dr Harrison has received honoraria for giving educational lectures or chairing scientific meetings from Bristol-Meyers Squibb, GlaxoSmithKline, Janssen, Lilly, Merck, Sanofi, and Servier pharmaceutical companies, and has been an adviser to Curidium, Janssen, and Wyeth. The other authors declare that, except for income received from the primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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