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IMMEDIATE COMMUNICATION

Sex differences in glutamate receptor gene expression in major depression and suicide

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Accumulating data indicate that the glutamate system is disrupted in major depressive disorder (MDD), and recent clinical research suggests that ketamine, an antagonist of the *N*-methyl-D-aspartate (NMDA) glutamate receptor (GluR), has rapid antidepressant efficacy. Here we report findings from gene expression studies of a large cohort of postmortem subjects, including subjects with MDD and controls. Our data reveal higher expression levels of the majority of glutamatergic genes tested in the dorsolateral prefrontal cortex (DLPFC) in MDD ($F_{21,59} = 2.32$, $P = 0.006$). *Posthoc* data indicate that these gene expression differences occurred mostly in the female subjects. Higher expression levels of GRIN1, GRIN2A-D, GRIA2-4, GRIK1-2, GRM1, GRM4, GRM5 and GRM7 were detected in the female patients with MDD. In contrast, GRM5 expression was lower in male MDD patients relative to male controls. When MDD suicides were compared with MDD non-suicides, GRIN2B, GRIK3 and GRM2 were expressed at higher levels in the suicides. Higher expression levels were detected for several additional genes, but these were not statistically significant after correction for multiple comparisons. In summary, our analyses indicate a generalized disruption of the regulation of the GluRs in the DLPFC of females with MDD, with more specific GluR alterations in the suicides and in the male groups. These data reveal further evidence that, in addition to the NMDA receptor, the AMPA, kainate and the metabotropic GluRs may be targets for the development of rapidly acting antidepressant drugs.

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INTRODUCTION

Major depressive disorder (MDD) is a common, recurring psychiatric disorder. The majority of individuals who die by suicide have a primary diagnosis of MDD.[1] MDD is characterized by cognitive disturbances, disrupted sleep, loss of pleasure and altered appetite. Conventional antidepressant treatments target the monoamine systems, but only one-third of patients achieve substantial improvement or remission after several weeks of treatment.[2] This time lag to therapeutic efficacy is a serious problem considering the high suicide risk associated with MDD.[1] This delay in treatment response also suggests that the primary pathophysiological mechanisms leading to MDD and suicide may lie outside the monoamine systems.

A growing body of data shows that abnormalities of the glutamate system lead to behaviors that correlate with psychiatric disorders, including MDD.[3] Glutamate is an excitatory neurotransmitter that is widely distributed in the brain, exerting its effects through the stimulation of several glutamate receptor (GluR) subtypes. These include the 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA), *N*-methyl-D-aspartate (NMDA), kainate (KAR) and metabotropic (mGluR) receptors.[4] Recent reports of a rapid antidepressant response in MDD patients after treatment with ketamine have led to increased scientific interest in the glutamate system and its possible dysfunction in MDD.[5–7] Unlike conventional antidepressant drugs, ketamine is an NMDA receptor (NMDAR) antagonist. It may exert its therapeutic effects after an acute low dose by increasing the synaptic release of glutamate.[8] Ketamine treatment produces antidepressant efficacy within hours of administration in 70% of treatment-resistant MDD patients. Symptom improvement usually lasts for a week or longer[9] but in general its therapeutic effects are short-lived.[10] Other glutamatergic agents, such as amantadine[11] and riluzole[12–14] are also potentially therapeutic in depression. Clinical studies suggest that the glutamatergic system contains several promising targets for improved antidepressant medications. Development of drugs for these novel targets requires an improved understanding of the role of the glutamate system in the pathophysiology of MDD.

The rapid antidepressant efficacy of ketamine may depend on increased glutamate neurotransmission through the activity of multiple GluR subtypes, possibly leading to optimal levels of synaptogenesis. Basic and clinical studies show that MDD is associated with altered activity in brain regions that are critical for the regulation of mood and cognition, including the dorsolateral prefrontal cortex (DLPFC).[15] Reduced numbers of synapses have been reported in the DLPFC of patients with MDD[16] and also in rodent models of stress. Conventional antidepressant drugs appear to reverse these neuronal deficits after a time delay, whereas ketamine rapidly induces synaptogenesis and reverses the synaptic deficits caused by chronic stress in animal models of depression. Converging evidence from basic, clinical and postmortem research reveals the existence of altered structure and activity within the DLPFC in MDD.

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MDD has a strong heritable component and has been associated with variation within the glutamatergic genes.[17–22] In addition to genetic variation, environmental factors such as early life adversity can influence gene expression.[23] Gene expression is a reflection of both genetic (inherited) and environmental precipitants of MDD. Investigation of postmortem brain is imperative to uncover important molecular abnormalities that contribute to the pathophysiology of MDD and suicide. Previous studies of GluR gene expression in the DLPFC have been conducted in small postmortem cohorts of MDD subjects and controls, using a variety of methods and yielding mixed results.[24–32] Nevertheless, these previous data indicate a fundamental dysfunction of the glutamate system in the frontal cortex in MDD. In the current study, we have tested the hypothesis that GluR gene expression is altered in the DLPFC in MDD in a large cohort of postmortem subjects from three diagnostic groups: MDD suicide, MDD non-suicide, and a group of comparison subjects with no history of psychiatric disorders. We report higher expression levels of a number of GluR genes in the DLPFC of MDD patients. These generalized differences in GluR expression were mostly driven by effects in the female groups, while there were fewer, more specific GluR expression differences in the male patients and in the suicides. Taken together, these data indicate that a disruption of the glutamate system occurs in the DLPFC of patients with MDD and in MDD patients who complete suicide. This disruption may be more severe in the female patients.

MATERIALS AND METHODS

Subjects

Frozen postmortem brain tissue from the DLPFC was obtained from two groups of subjects: (1) patients diagnosed by Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria with MDD; and (2) a comparison group (CTRL) with no history of psychiatric, substance abuse or neurological disorders. Specimens were obtained from the Clinical Brain Disorders Branch at the National Institute of Mental Health. Brains were collected from the Offices of the Chief Medical Examiner in Washington, DC and in Northern Virginia, USA. Supplementary Table S1 summarizes the demographic variables of the postmortem subjects. Every case had a 20-item telephone screening completed by a physician on the day of donation to gather information on medical, psychiatric, substance abuse and social history. Psychiatric records and/or family informant interviews (computer-assisted Structured Clinical Interview for DSM-IV Disorders and NIMH Psychological Autopsy interview) were conducted with all psychiatric cases, when possible, by a master's level clinician. Psychiatric narrative summaries were prepared for every case, incorporating all of the above information, and two board-certified psychiatrists reviewed these cases to arrive at lifetime DSM-IV diagnoses. Postmortem interval (PMI) was calculated as time elapsed between death and tissue freezing, in hours.

Toxicological screenings were performed for every case by the medical examiner's office in the blood, brain or other available matrix to screen for prescribed medications, drugs of abuse (ethanol, cocaine and metabolites, opiates, phencyclidine) and other substances that may have been related to the cause of death. We have omitted postmortem subjects who died from any form of poisoning, for example, overdose and carbon monoxide poisoning. To measure the pH of the brain, 100 mg of homogenized frozen brain tissue was mixed with 1 ml of cooled deionized water and pH was measured using a FiveEasy Plus pH meter (Mettler-Toledo LLC, Columbus, OH, USA).

Autopsy information was reviewed, including cause and manner of death (see Supplementary Table S1), to exclude cases with hepatic or renal disease (which may cause increases in astroglia). We have also omitted postmortem subjects who died from any form of poisoning, for example, overdose or carbon monoxide poisoning, from any of the reported analyses. Macroscopic and microscopic examination of the brain (M.M.H.), including Bielschowsky's silver stain (adapted for paraffin sections) on multiple cortical areas, was used to exclude cases with neuritic pathology, such as Alzheimer's disease or cerebrovascular accidents. All layers of the cortex were removed. The middle frontal gyrus incorporating BA9/46 was dissected from a 1 cm thick slab just rostral to the rostrum of the corpus callosum, following the maps generated by Rajkowska and Goldman-Rakic.[33]

Preparation of cDNA

The laboratory personnel were blind to clinical data during the experiments. RNA was extracted from gray matter by standard methods as previously described.[34] The RNA integrity number (RIN) was determined using an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) to provide a measure of RNA quality, and the average RIN value was 8.3 (Supplementary Table S1). Complementary DNA (cDNA) was synthesized from equal quantities of RNA from each subject by the reverse transcriptase reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Aliquots of cDNA from each subject were pooled for use as standards. Dilutions of 1:20, 1:10, 1:5, and undiluted pooled cDNA were used to generate a calibration curve according to the relative standard curve method for gene expression analysis (http://www.AppliedBiosystems.com). All RNA samples were diluted to 20 ng per microliter, followed by preamplification using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Preamplification of cDNA was necessary due to the low starting concentration of mRNA. Equal volumes of each TaqMan assay to be used for expression analysis were combined for the preamplification reaction. Forty microliter cDNA, together with 4 µl pooled assays and 44 µl TaqMan JumpStart (Sigma-Aldrich, St Louis, MO, USA), was preamplified for 14 cycles as previously described.[35,36]

Gene expression assays

Gene expression was measured using Applied Biosystems assays (https://products.appliedbiosystems.com), which are listed in Supplementary Table S2. MGluR6 and mGluR8 were not expressed at detectable levels in the DLPFC, as predicted by previous studies.[37] Quantitative PCR assays for each target gene were performed in duplicate on cDNA samples in optically clear plates using the Applied Biosystems ViiA 7 System (Life Technologies, Grand Island, NY, USA). All real-time PCR data were captured using the ViiA 7 software v.1.1 (Life Technologies). For every sample, an amplification plot was generated, showing the increase in the reporter dye fluorescence with each cycle of PCR. The 'housekeeping' genes used to normalize the gene expression data were tested by analysis of variance for association with independent variables and covariates. Any housekeeping gene that was significantly associated with diagnosis, sex or more than one demographic variable, was eliminated from further analyses (Supplementary Table S4). Of the six housekeeping genes measured, three (GUSB, B2M, ACTB) were included in our analyses. The geometric mean of the expression of these genes was not associated with diagnosis or sex, nor was there an interaction of sex by diagnosis ($P > 0.25$). These genes were used as standards with which we calculated ratios of gene expression for each test transcript. The geometric mean of their expression levels was used for normalization, because averaging of multiple control genes has greater accuracy than calculations from a single housekeeping gene. The relative expression levels of test transcripts were calculated using the Relative Standard Curve Method (www.AppliedBiosystems.com).

Statistical analyses

We hypothesized that glutamatergic dysfunction occurs in MDD. This was tested by examining the expression of GluR genes in the DLPFC of a postmortem cohort including patients with diagnoses of MDD ($n = 53$) and a comparison group of psychiatrically healthy subjects (controls, $n = 32$). The postmortem cohort tested (total $n = 85$) had sufficient power to detect a large effect (Cohen's $D = 0.8$) when MDD and controls were compared. There was sufficient power to test for an effect of each sex by diagnosis when male MDD patients ($n = 26$) were compared with male controls ($n = 19$) and when female patients ($n = 27$) were compared with female controls ($n = 13$). There was also sufficient power to detect a large effect when the MDD-suicide (MDD-S, $n = 34$) was compared with MDD-non-suicide (MDD-NS, $n = 19$). The sample had 50% power to detect a large effect when comparing the males and females separately for suicide completion. Shapiro–Wilk tests were used to determine if data were normally distributed and Levene's test of homogeneity of variance was used to confirm that the variance in each group compared was similar. Normally distributed data were analyzed using multivariate analysis of covariance (ANCOVA) to investigate main effects, and *posthoc* analyses of individual genes were performed using univariate ANCOVA, with PMI, age at death, RNA integrity number and brain pH included as covariates. Analyses included relative gene expression as the dependent variable and diagnosis as the independent variable (Tables 1a and b). The false discovery method was used to correct for the effects of multiple comparisons.[38] The statistical relationship between gene expression and demographic variables was also tested using analysis of variance and Pearson correlation analyses (Supplementary Table S3). Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp., Armonk, NY, USA).

The following models were tested using ANCOVA:

1. Gene expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Diagnosis}$
2. Gene expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Diagnosis}+\beta6\text{Sex}+\beta7\text{Sex*Diagnosis}$

with Diagnosis as a two-level factor, Control vs MDD.

Subsequently, we compared GluR gene expression between subgroups of the MDD cases to test for potential predictors of suicide. The model below was tested separately in males and females:

3. Gene expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Suicide}$

with Suicide as a two-level factor, MDD-S vs MDD-NS.

We then tested the hypothesis that the relative expression of higher sensitivity vs lower sensitivity NMDA receptor subunits is altered in suicide. NMDARs containing GluN2A and GluN2B protein subunits display a higher sensitivity compared with NMDARs containing either GluN2C or GluN2D. Therefore, we tested the ratio of GluN2C and GluN2D expression to GluN2A and GluN2B (GRIN2C*GRIN2D)/(GRIN2A*GRIN2B) in the patients, as a potential predictor of suicide, in males and females separately.

4. Expression (GRIN2C*GRIN2D)/(GRIN2A*GRIN2B) $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Suicide}$

with Suicide as a two-level factor, MDD-S vs MDD-NS.

Posthoc analyses also included tests of the potential effects of alcohol, antidepressant drug or smoker status on gene expression in our postmortem MDD subjects using ANCOVA:

5. Gene expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Alcohol}+\beta6\text{X}+\beta7\text{Alcohol*X}$

with Alcohol as a two-level factor in the MDD group, positive vs negative and X is Diagnosis (MDD vs controls) or Suicide (MDD-S vs MDD-NS). Of the MDD subjects who were tested for the presence of alcohol postmortem, 7 were positive and 24 were negative.

6. Gene expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Antidepressant}+\beta6\text{X}+\beta7\text{Antidepressant*X}$

with Antidepressant as a two-level factor in the MDD group, positive vs negative and X is Diagnosis (MDD vs controls) or Suicide (MDD-S vs MDD-NS). Of the MDD subjects who were tested for the presence of antidepressant drugs postmortem, 24 were positive and 12 were negative.

7. Gene expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Smoker status}+\beta6\text{X}+\beta7\text{Smoker status*X}$

with Smoker status as a two-level factor in the MDD group, smoker vs non-smoker and X is Diagnosis (MDD vs controls) or Suicide (MDD-S vs MDD-NS). Of the MDD subjects, 19 were smokers and 23 were non-smokers at their time of death.

Only four subjects were found to be positive for antipsychotic drugs in toxicological analyses, and therefore the effect of antipsychotics could not be tested in our cohort.

Posthoc tests were conducted in female subjects to test the expression of the vesicular glutamate transporter (VGlut1), which is considered to be a marker of glutamatergic neurons in the prefrontal cortex.[39]

8. VGlut1 expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Age}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{X}$

with X as a two-level factor. X is Diagnosis (MDD vs controls) or Suicide (MDD-S vs MDD-NS).

Posthoc tests were also conducted in female subjects to test the expression of the glutamatergic genes in putative premenopause vs postmenopausal age groups, with age at menopause assumed at 51 years, as indicated by the National Institute of Aging.

9. Gene expression $(y) = \beta0+\beta1\text{PMI}+\beta2\text{Menopause}+\beta3\text{pH}+\beta4\text{RIN}+\beta5\text{Diagnosis}+\beta6\text{Menopause*Diagnosis}$

with Menopause as a two-level factor, premenopause (< 51 years) vs postmenopausal age (≥ 51 years) and Diagnosis as a two-level factor, control vs MDD.

RESULTS

Analyses of GluR gene expression in our postmortem cohort reveal increased expression of almost every GluR gene tested in MDD (Figure 1a). General linear model multivariate analyses were used to compare GluR gene expression in the MDD patients relative to the controls and also in the MDD non-suicide cases relative to the MDD suicides. These analyses revealed higher levels of GluR gene expression in the MDD patients relative to the controls ($F_{21,59} = 2.32$, $P = 0.006$). *Posthoc* data indicate that 5 of the 21 GluR genes tested had statistically significant differences in mRNA abundance in MDD after using the FDR method[38] to correct for multiple comparisons (Table 1a). The FDR threshold for significance was calculated to be $P \leqslant 0.02$.

The incidence of MDD and suicide is reported to differ between males and females.[40,41] Therefore, *posthoc* analyses were conducted to test for sex by diagnosis interactions. These analyses revealed that many of our findings were driven by differences between the female groups. Five of the six genes encoding NMDAR subunits and three of the four genes encoding the AMPAR subunits had higher expression in the female MDD group (Figure 2a). Of the KAR subunits, GRIK1 and GRIK2 had significantly higher expression in females with MDD, after correction for multiple comparisons. Analyses of the mGluRs revealed that four

Table 1a. Glutamate receptor gene expression is altered in the dorsolateral prefrontal cortex in major depression

Gene	*Diagnosis*					*CTRL vs MDD*			*Sex × Diagnosis*	
			Mean	*s.d.*	N	*Mean diff*	$F_{1,79}$	P	$F_{1,77}$	P
GRIA1	CTRL	M	1.77	0.57	19	−0.191	2.6	0.11	2.7	0.11
		F	1.50	1.03	13					
	MDD	M	1.76	0.45	26					
		F	1.94	0.41	27					
GRIA2	CTRL	M	1.14	0.22	19	−0.206	10.1	**0.002**	10.2	**0.002**
		F	0.89	0.40	13					
	MDD	M	1.15	0.29	26					
		F	1.33	0.26	27					
GRIA3	CTRL	M	1.22	0.25	19	−0.157	3.0	0.09	8.4	**0.005**
		F	0.96	0.41	13					
	MDD	M	1.19	0.34	26					
		F	1.34	0.24	27					
GRIA4	CTRL	M	1.26	0.29	19	−0.150	2.7	0.10	12.4	**0.001**
		F	0.83	0.48	13					
	MDD	M	1.14	0.40	26					
		F	1.33	0.35	27					
GRIN1	CTRL	M	1.26	0.38	19	−0.163	2.4	0.13	13.6	**0.0004**
		F	0.82	0.45	13					
	MDD	M	1.18	0.29	26					
		F	1.31	0.28	27					
GRIN2A	CTRL	M	1.25	0.42	19	−0.123	2.0	0.16	9.8	**0.002**
		F	0.85	0.56	13					
	MDD	M	1.14	0.28	26					
		F	1.28	0.34	27					
GRIN2B	CTRL	M	1.15	0.35	19	−0.140	3.1	0.08	4.8	0.03
		F	0.86	0.51	13					
	MDD	M	1.09	0.50	26					
		F	1.25	0.36	27					
GRIN2C	CTRL	M	1.26	0.59	19	−0.069	0.0	0.89	6.6	**0.01**
		F	0.86	0.48	13					
	MDD	M	1.12	0.47	26					
		F	1.21	0.36	27					
GRIN2D	CTRL	M	1.31	0.42	19	−0.085	1.3	0.25	13.6	**0.0004**
		F	0.81	0.42	13					
	MDD	M	1.14	0.31	26					
		F	1.25	0.36	27					
GRIN3A	CTRL	M	1.38	0.69	19	0.023	0.0	0.86	7.8	**0.01**
		F	0.85	0.67	13					
	MDD	M	1.10	0.41	26					
		F	1.18	0.44	27					
GRIK1	CTRL	M	0.95	0.56	19	−0.124	3.7	0.06	3.6	0.06
		F	0.81	0.56	13					
	MDD	M	0.85	0.41	26					
		F	1.17	0.56	27					
GRIK2	CTRL	M	0.98	0.20	19	−0.197	12.5	**0.001**	14.1	**0.0003**
		F	0.78	0.27	13					
	MDD	M	1.02	0.22	26					
		F	1.18	0.16	27					
GRIK3	CTRL	M	1.21	0.36	19	0.152	0.1	0.79	0.1	0.76
		F	1.37	1.51	13					
	MDD	M	1.09	0.34	26					
		F	1.15	0.39	27					
GRIK4	CTRL	M	1.00	0.27	19	−0.006	0.3	0.61	4.5	0.04
		F	0.85	0.42	13					
	MDD	M	0.89	0.17	26					
		F	1.00	0.22	27					
GRIK5	CTRL	M	1.42	0.36	19	−0.043	0.2	0.62	2.9	0.09
		F	1.28	0.77	13					
	MDD	M	1.34	0.30	26					
		F	1.46	0.30	27					
GRM1	CTRL	M	1.18	0.38	19	−0.121	4.6	0.04	8.6	**0.004**
		F	0.78	0.51	13					
	MDD	M	1.08	0.34	26					
		F	1.19	0.31	27					
GRM2	CTRL	M	1.15	0.43	19	−0.007	0.6	0.45	4.5	0.04
		F	0.95	0.62	13					
	MDD	M	0.98	0.27	26					
		F	1.16	0.43	27					

Table 1a. (Continued)

Gene	*Diagnosis*					*CTRL vs MDD*			*Sex × Diagnosis*	
			Mean	*s.d.*	N	*Mean diff*	$F_{1,79}$	P	$F_{1,77}$	P
GRM3	CTRL	M	0.90	0.23	19	0.105	1.6	0.21	0.0	0.82
		F	0.91	0.29	13					
	MDD	M	0.77	0.24	26					
		F	0.83	0.24	27					
GRM4	CTRL	M	0.95	0.24	19	−0.131	6.1	**0.016**	4.4	0.04
		F	0.71	0.42	13					
	MDD	M	0.97	0.15	26					
		F	1.00	0.23	27					
GRM5	CTRL	M	0.70	0.16	19	−0.033	1.4	0.24	14.7	**0.0003**
		F	0.50	0.30	13					
	MDD	M	0.59	0.15	26					
		F	0.72	0.19	27					
GRM7	CTRL	M	0.90	0.17	19	−0.182	7.7	**0.007**	12.4	**0.0007**
		F	0.66	0.32	13					
	MDD	M	0.93	0.25	26					
		F	1.03	0.16	27					

Abbreviations: CTRL, control; F, female; M, male; MDD, major depressive disorder; mean diff, difference in the means of the groups compared. Gene expression data are summarized for all diagnostic groups. *Posthoc* tests were performed to compare expression levels in CTRL subjects with MDD subjects. Sex by diagnosis interactions were tested for each gene. The demographic variables are summarized in Supplementary Table S1. Data were corrected for multiple comparisons using the false discovery rate (FDR) method as described in the Methods. Statistically significant differences between the groups after FDR correction are indicated in bold font.

Table 1b. Glutamate receptor gene expression is altered in the dorsolateral prefrontal cortex of major depressive suicides

Gene	*Suicide*					*MDD-NS* vs *MDD-S*			
			Mean	*s.d.*	N		*Mean diff*	*F*	P
GRIA1	MDD-S	M	1.76	0.42	16	M	0.01	0.06	0.81
		F	2.02	0.43	18	F	0.24	1.82	0.19
	MDD-NS	M	1.75	0.51	10	T	0.13	0.47	0.50
		F	1.78	0.3	9				
GRIA2	MDD-S	M	1.14	0.31	16	M	−0.03	0.17	0.69
		F	1.36	0.27	18	F	0.10	0.51	0.48
	MDD-NS	M	1.17	0.27	10	T	0.04	0.03	0.86
		F	1.27	0.24	9				
GRIA3	MDD-S	M	1.19	0.34	16	M	−0.01	0.01	0.92
		F	1.37	0.26	18	F	0.07	0.83	0.37
	MDD-NS	M	1.2	0.37	10	T	0.04	0.23	0.64
		F	1.3	0.21	9				
GRIA4	MDD-S	M	1.19	0.42	16	M	0.13	0.19	0.67
		F	1.37	0.39	18	F	0.12	0.09	0.76
	MDD-NS	M	1.06	0.38	10	T	0.14	0.43	0.51
		F	1.25	0.26	9				
GRIN1	MDD-S	M	1.15	0.32	16	M	−0.07	0.20	0.66
		F	1.33	0.29	18	F	0.08	0.21	0.65
	MDD-NS	M	1.22	0.26	10	T	0.01	0.00	0.95
		F	1.25	0.24	9				
GRIN2A	MDD-S	M	1.17	0.28	16	M	0.09	0.17	0.68
		F	1.33	0.36	18	F	0.15	0.47	0.50
	MDD-NS	M	1.08	0.28	10	T	0.13	0.59	0.45
		F	1.18	0.27	9				
GRIN2B	MDD-S	M	1.18	0.57	16	M	0.22	0.47	0.50
		F	1.38	0.36	18	F	0.38	7.30	**0.01**
	MDD-NS	M	0.96	0.37	10	T	0.31	3.88	0.05
		F	1	0.19	9				
GRIN2C	MDD-S	M	1.01	0.27	16	M	−0.29	1.21	0.28
		F	1.14	0.39	18	F	−0.21	3.84	0.06
	MDD-NS	M	1.3	0.66	10	T	−0.24	2.54	0.12
		F	1.35	0.27	9				
GRIN2D	MDD-S	M	1.13	0.36	16	M	−0.01	0.03	0.88
		F	1.32	0.4	18	F	0.21	0.79	0.38
	MDD-NS	M	1.14	0.22	10	T	0.11	0.30	0.59
		F	1.11	0.23	9				

Table 1b. (Continued)

Gene	*Suicide*					*MDD-NS* vs *MDD-S*			
			Mean	*s.d.*	N		*Mean diff*	*F*	P
GRIN3A	MDD-S	M	1.16	0.38	16	M	0.16	0.15	0.70
		F	1.28	0.5	18	F	0.30	1.23	0.28
	MDD-NS	M	1	0.47	10	T	0.24	1.07	0.31
		F	0.98	0.18	9				
GRIK1	MDD-S	M	0.93	0.47	16	M	0.18	0.69	0.42
		F	1.34	0.6	18	F	0.51	3.46	0.08
	MDD-NS	M	0.74	0.26	10	T	0.36	3.89	0.05
		F	0.83	0.28	9				
GRIK2	MDD-S	M	1.02	0.24	16	M	0.02	0.00	0.97
		F	1.17	0.16	18	F	−0.03	0.12	0.73
	MDD-NS	M	1.01	0.21	10	T	0.00	0.01	0.92
		F	1.2	0.19	9				
GRIK3	MDD-S	M	1.24	0.25	16	M	0.38	9.85	**0.01**
		F	1.29	0.39	18	F	0.41	9.14	**0.01**
	MDD-NS	M	0.85	0.34	10	T	0.40	14.64	**0.0004**
		F	0.88	0.23	9				
GRIK4	MDD-S	M	0.93	0.15	16	M	0.11	1.14	0.30
		F	1.04	0.22	18	F	0.12	0.24	0.63
	MDD-NS	M	0.82	0.18	10	T	0.12	1.30	0.26
		F	0.92	0.19	9				
GRIK5	MDD-S	M	1.32	0.25	16	M	−0.05	0.02	0.88
		F	1.51	0.34	18	F	0.15	0.54	0.47
	MDD-NS	M	1.37	0.38	10	T	0.06	0.33	0.57
		F	1.36	0.18	9				
GRM1	MDD-S	M	1.15	0.28	16	M	0.16	0.59	0.45
		F	1.21	0.34	18	F	0.06	0.00	0.95
	MDD-NS	M	0.98	0.42	10	T	0.12	0.36	0.55
		F	1.15	0.26	9				
GRM2	MDD-S	M	1.04	0.28	16	M	0.14	0.91	0.35
		F	1.31	0.4	18	F	0.45	6.63	**0.02**
	MDD-NS	M	0.89	0.22	10	T	0.31	6.03	**0.02**
		F	0.86	0.31	9				
GRM3	MDD-S	M	0.79	0.26	16	M	0.07	0.00	0.96
		F	0.86	0.24	18	F	0.09	0.00	0.98
	MDD-NS	M	0.72	0.19	10	T	0.08	0.01	0.91
		F	0.77	0.24	9				
GRM4	MDD-S	M	1.01	0.14	16	M	0.09	1.03	0.32
		F	1.07	0.18	18	F	0.21	4.96	0.04
	MDD-NS	M	0.91	0.17	10	T	0.15	4.86	0.03
		F	0.86	0.28	9				
GRM5	MDD-S	M	0.61	0.12	16	M	0.06	0.13	0.72
		F	0.74	0.2	18	F	0.07	0.14	0.71
	MDD-NS	M	0.55	0.19	10	T	0.08	0.17	0.68
		F	0.67	0.17	9				
GRM7	MDD-S	M	0.88	0.26	16	M	−0.13	1.07	0.31
		F	1.05	0.17	18	F	0.04	0.22	0.64
	MDD-NS	M	1.01	0.22	10	T	−0.04	0.16	0.69
		F	1	0.15	9				

Abreviations: F, female; MDD-NS, major depressive disorder non-suicide; MDD-S, major depressive disorder suicide; mean diff, difference in the means of the groups compared; M, male; T, total. Gene expression data are summarized for all the diagnostic groups. *Posthoc* tests were performed to compare the expression levels between MDD-NS and MDD-S cases, as described in the Methods. The demographic variables for the subjects are summarized in Supplementary Table S1. Degrees of freedom were 1, 46 for all subjects when MDD-S vs MDD-NS were compared. Comparisons of males and females separately had degrees of freedom 1, 20 for male MDD-S vs MDD-NS and 1, 21 for female MDD-NS vs MDD-S. These separate analyses of suicide in males and females had only 50% power to detect large effects, while analyses of the total sample had sufficient statistical power to detect large effects. Data were corrected for multiple comparisons using the FDR method as described in the Methods. Statistically significant differences between the groups after FDR correction are indicated in bold font.

of the six genes tested, GRM1, GRM4, GRM5 and GRM7, showed significantly higher expression in females with MDD after correction for multiple comparisons (Figure 2a). However, in males, the only significant difference was the relatively lower level of GRM5 expression in the male patients with MDD (Figure 3a). MGluR6 and mGluR8 were not detected in the DLPFC and therefore could not be analyzed.

To identify predictors of suicide risk within MDD, we have tested for differences in gene expression between the MDD suicides (MDD-S) and MDD non-suicides (MDD-NS). Higher expression levels of GRIN2B, GRIK3 and GRM2 were noted in the MDD-S group (Table 1b, Figure 1b) but only GRIK3 ($F_{1,46} = 14.6$, $P = 0.0004$) and GRM2 ($F_{1,46} = 6.03$, $P = 0.018$) reached statistical significance when corrected for multiple comparisons. Relatively higher

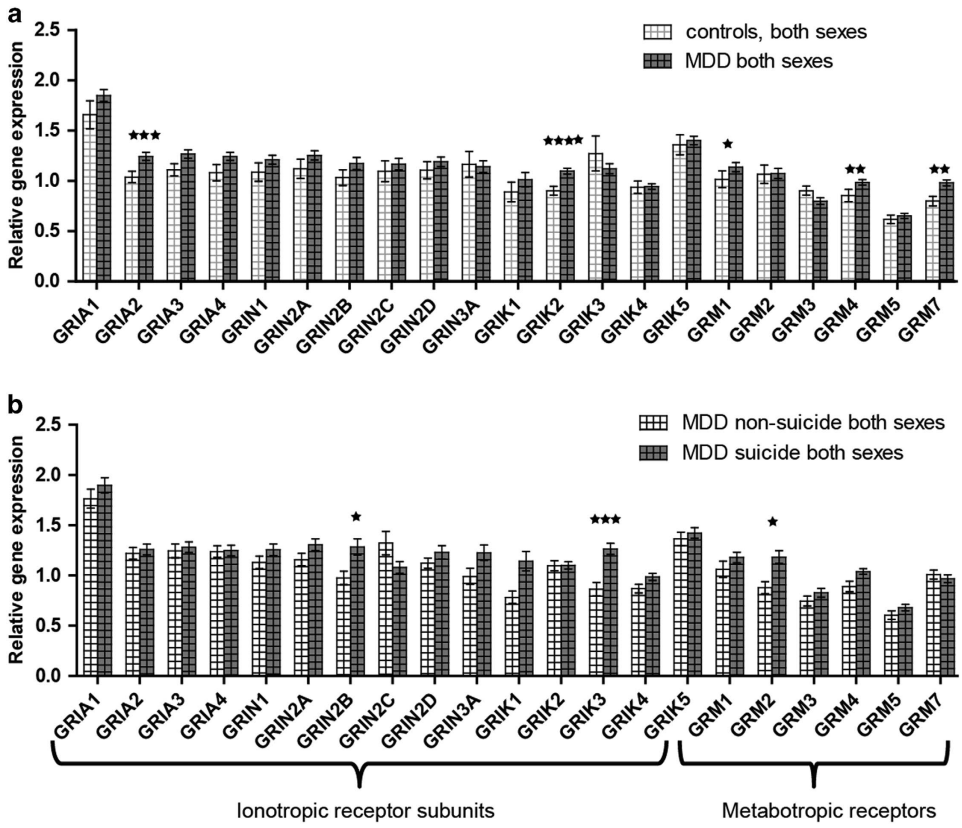

Figure 1. Higher levels of expression of glutamate receptor genes occur in the dorsolateral prefrontal cortex in major depressive disorder (MDD) and suicide. (**a**) *Posthoc* analyses of gene expression differences between controls and the MDD subjects reveal that 5 of the 21 glutamate receptor genes tested had higher expression in the MDD group. (**b**) *Posthoc* analyses reveal that three genes analyzed had significantly altered expression when we compared MDD subjects who died by suicide and MDD subjects who did not die by suicide. Data were corrected for multiple comparisons using the false discovery rate method. Statistically significant differences are indicated with asterisks in the figure. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.00005$. GRIA, AMPAR subunit; GRIK, KAR subunit; GRM, metabotropic glutamate receptor subtype; GRIN, NMDAR subunit.

expression levels of GRIK3 in the suicide group were consistent in both sexes (Figures 1b, 2b and 3b), but the other findings, including higher levels of GRIN2B in the suicide cases, were the greatest in the female groups (Figures 1b and 2b). These comparisons within subgroups of the MDD patients are likely to minimize the potential confounds of medication history and substance abuse that can create differences between MDD patients and control groups.

Posthoc tests of the expression of the glutamate cell marker in the cortex, VGlut1,[39] revealed that female MDD patients had higher levels of VGlut1 expression in the DLPFC ($F_{1,34} = 6.53$, $P = 0.02$) relative to female controls and that there was a similar trend in female MDD-S relative to female MDD-NS ($F_{1,21} = 3.85$, $P = 0.06$). VGlut1 expression was not associated with antidepressant positive vs negative status. The NMDAR is composed of four GluN subunits. Variation in the subunit composition of the NMDAR alters the activity of the receptor. NMDARs containing GluN2A and GluN2B protein subunits display a higher sensitivity to voltage-dependent Mg^{2+} block, higher Ca^{2+} permeability and higher single-channel conductance than NMDARs containing either GluN2C or GluN2D.[42–45] Therefore, we tested the ratio of GluN2C and GluN2D expression to GluN2A and GluN2B (GRIN2C*GRIN2D)/(GRIN2A*GRIN2B) in the patient cohort. In the female subjects, the MDD-suicides had higher expression levels of the high-sensitivity NMDAR subunits compared with the non-suicides leading to a decrease in this ratio ($F_{1,21} = 9.38$, 1 df, $P = 0.006$). This ratio was not associated with antidepressant/alcohol or smoker status of the MDD subjects.

We tested for differences in GluR gene expression in the recurrent depressed subjects ($n = 46$) relative to the single-episode patients ($n = 7$) and found no significant differences between these groups for any of the genes tested. Moreover, subjects with recurrent depression with psychosis ($n = 13$) were compared with subjects who had recurrent depression without psychosis ($n = 33$), and no significant differences in GluR gene expression were detected. No information about treatment response in these subjects is available.

The gene expression differences between males and females were not associated with sex differences of potential confounding variables that we could test: alcohol, antidepressant drugs, or cigarette smoking. Chi-square analyses revealed no significant difference in the frequencies of smoker status, antidepressant status or alcohol status between the MDD males and MDD females ($P > 0.3$).

GRIK1 expression was higher in non-smokers of both control and MDD groups ($F_{1,65} = 6.91$, $P = 0.01$) but there was no interaction between smoker status and diagnosis or suicide. Antidepressant status was not associated with the expression of any glutamatergic gene in either males or females. There were too few subjects who were positive for alcohol at death to test effects in the different sexes. When all MDD subjects were analyzed, no single glutamatergic gene showed significantly altered expression in the alcohol positive subjects. Only four subjects were found to

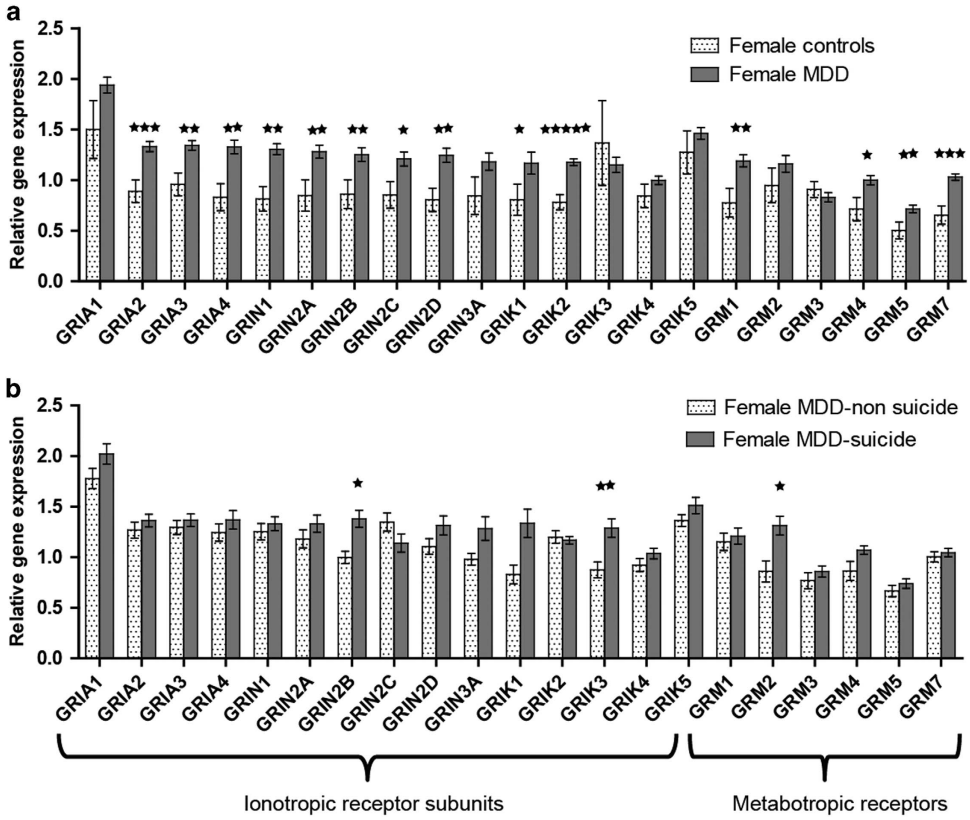

Figure 2. Females with major depressive disorder (MDD) exhibit generalized differences in the expression of glutamate receptor genes in the dorsolateral prefrontal cortex. (**a**) Female MDD patients relative to female controls showed higher levels of expression of 19 of the 21 genes tested. Of these, 14 genes showed statistically significant differences in expression, which are indicated by asterisks. (**b**) Comparisons of female MDD suicides with female non-suicides reveal that three glutamate receptor genes had significantly higher expression in the female subjects who died by suicide. Higher levels of expression were detected for GRIN2B, GRIK3 and GRM2 ($P < 0.02$). Data were corrected for multiple comparisons using the false discovery rate method. Statistically significant differences after correction are indicated with asterisks. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.00005$. GRIA, AMPA receptor subunit; GRIK, kainate receptor subunit; GRM, metabotropic glutamate receptor subtype; GRIN, NMDA receptor subunit.

be positive for antipsychotic drugs in toxicological analyses, and therefore the effect of antipsychotics could not be tested in our cohort.

Analyses of gene expression were conducted to compare the female groups < 51 years (9 controls, 18 MDD) relative to females aged $\geqslant 51$ years (5 controls, 9 MDD). Age group by diagnosis interactions were detected for the expression levels of GRIN2C and GRM3. In the case of GRIN2C, lower levels of expression were observed in the female controls aged < 51 years compared with all other groups ($F_{1,33} = 10.8$, $P = 0.002$). In contrast, lower levels of GRM3 expression were observed in the MDD group aged > 51 years relative to the other groups ($F_{1,33} = 6.0$, $P = 0.02$). If corrected for multiple comparisons, only GRIN2C showed significant differences in gene expression.

DISCUSSION

We have detected differences in the expression of GluR genes in the DLPFC of subjects with MDD, and in MDD with completed suicide. The majority of the 21 GluR genes that were tested showed higher levels of expression in the MDD subjects relative to controls. Of these, five genes showed statistically significant differences after FDR correction for multiple comparisons. The greatest effects were detected in the female groups. Previous studies have not included large numbers of females with MDD, and this may explain why similar effects have not been previously reported. This pattern of gene expression was not observed in our analyses of genes encoding monoamine transporters in a separate study being conducted on this postmortem cohort (Bristow *et al*., in preparation). Therefore, this gene expression signature may occur in specific cell populations of the DLPFC in MDD and suicide.

Our data could indicate that there is a general abnormality in the function of glutamatergic cells in the DLPFC of females with MDD. Indeed a preliminary analysis of the glutamatergic cell marker, VGlut1, revealed that female MDD subjects had higher levels of expression relative to the female controls. A similar trend was observed in female MDD suicides relative to female MDD non-suicides. These results were not associated with the presence of antidepressant drugs postmortem. These preliminary findings indicate that there may be higher numbers of glutamate neurons in the DLPFC of females with MDD, but this requires investigation in future studies.

Our data show that females with MDD have higher expression levels of all NMDAR genes, and only GRIN3A (GluN3A, NR3A) failed to meet the threshold for statistical significance. These data may indicate a dysfunction of NMDAR activity in females with MDD. The expression of GRIN2B (GluN2B, NR2B) was higher in all the MDD patients who died by suicide relative to the MDD patients who did not die by suicide. Therefore GRIN2B mRNA levels may be biological markers of suicide. Indeed, genetic polymorphisms of GRIN2B have been reported to predict treatment resistance in MDD,[46] suicide attempts[47] and reasoning ability.[48] These GRIN2B polymorphisms, and/or other polymorphisms in linkage

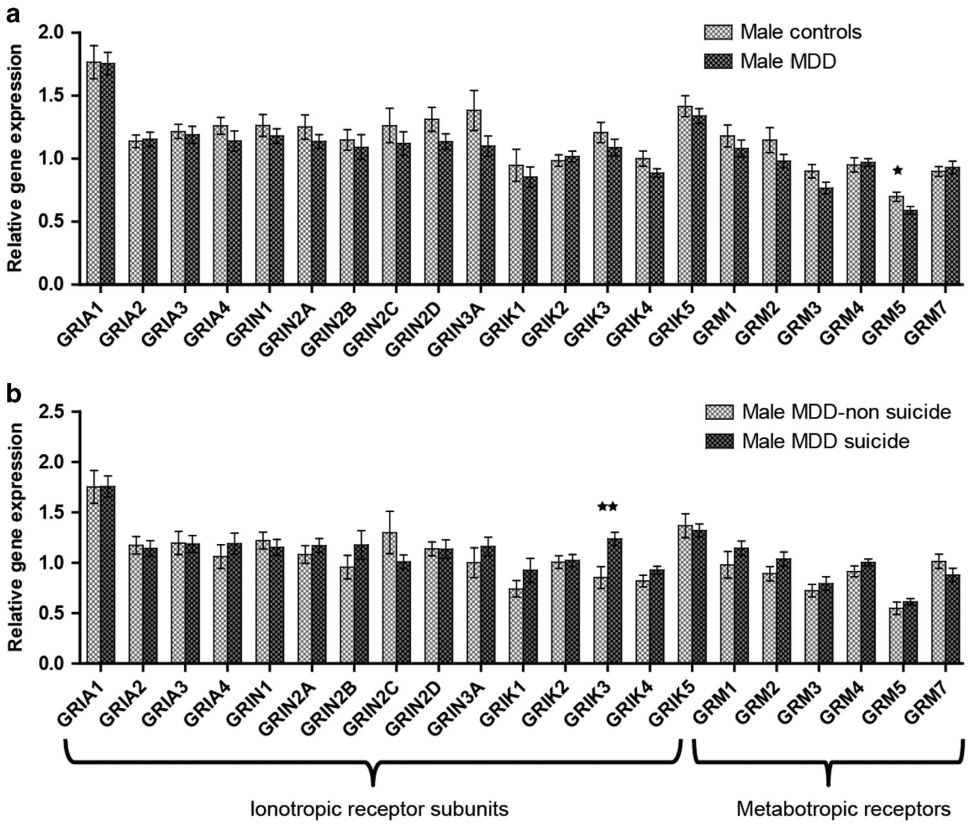

Figure 3. Males with major depressive disorder (MDD) exhibit specific differences in the expression of glutamate receptor genes in the dorsolateral prefrontal cortex. In comparison with the female groups, fewer gene expression differences occurred between the male diagnostic groups. *Posthoc* data are summarized. Data were corrected for multiple comparisons using the false discovery rate as described in the Methods. (**a**) Male MDD patients have lower expression of GRM5 relative to male controls ($P < 0.02$). (**b**) GRIK3 had significantly higher expression in male MDD subjects who died by suicide relative to male MDD subjects who did not die by suicide. $^{*}P < 0.05$, $^{**}P < 0.005$. GRIA, AMPA receptor subunit; GRIK, kainate receptor subunit; GRM, metabotropic glutamate receptor subtype; GRIN, NMDA receptor subunit.

disequilibrium, may alter GRIN2B mRNA abundance and are potential biomarkers for suicide.

There was a significantly lower ratio of GRIN2C*GRIN2D to GRIN2A*GRIN2B expression in MDD suicide subjects. These data may indicate differential expression of NMDAR subtypes in suicide. Physiological studies show that NMDARs containing GluN2A (GRIN2A) or GluN2B (GRIN2B) subunits display a higher sensitivity to voltage-dependent Mg^{2+} block, higher Ca^{2+} permeability and higher single-channel conductance than NMDARs containing GluN2C (GRIN2C) or GluN2D (GRIN2D).[42–45] GluN2A and GluN2B are the NMDAR subunits predominantly responsible for synaptic plasticity in the PFC.[49] Our findings of decreased expression of GRIN2C and GRIN2D relative to GRIN2A and GRIN2B may indicate that there was greater synaptic plasticity in the DLPFC of MDD suicides compared with MDD subjects who did not die by suicide. Synaptic NMDAR activation is also associated with neuroprotection, whereas stimulation of extra-synaptic NMDARs, which are enriched with GluN2B subunits, initiates cell destruction pathways. Extra-synaptic NMDARs also oppose the effects of synaptic NMDARs in the determination of neuronal cell fate.[50] Therefore, increased expression of the extra-synaptic NMDAR subunits may indicate reduced neuroprotection and/ or neurogenesis in MDD. Indeed, extra-synaptic GluN2B-containing NMDARs have been targeted for antidepressant drug development, and selective antagonism of GluN2B appears to produce a significant antidepressant effect in patients with treatment-resistant MDD.[51] Our data indicate that females with MDD who are at high risk of suicide may have the greatest benefit from this therapeutic strategy.

In contrast with the females, only one gene showed significant association with diagnosis in the male groups. Lower levels of GRM5 expression were observed in the male MDD patients relative to male controls. GRM5 encodes mGluR5, which has previously been reported to have reduced expression in the prefrontal cortices of MDD subjects, in a postmortem group in which 80% of the subjects were male.[30] Therefore, our findings in the male subjects appear to replicate these data. Interestingly, mGluR5 knockout (male) mice have reduced levels of depressive behaviors relative to controls, indicating that mGluR5 activity is depressive.[52] Moreover, negative allosteric modulators of mGluR5 have been found to have antidepressant properties.[53] It is possible that lower expression of GRM5 in males with MDD may not be correlated with lower mGluR5 activity and/or expression levels of GRM5 may not be lower in other brain regions. In contrast with the male groups, females with MDD had higher GRM5 expression than female controls, so our data may indicate that mGluR5 negative allosteric modulators will be more effective antidepressants in female patients.

The genes encoding non-NMDA ionotropic receptors were also associated with diagnosis and suicide in our cohort. The AMPAR subunits GluA2-4 showed significantly higher expression in the female MDD patients. Our data are consistent with previously reported data indicating a trend for increased NMDAR and AMPAR subunit expression in the DLPFC of a smaller group of MDD

subjects.[54] Indeed, it has been suggested that the antidepressant effects of ketamine are dependent on the enhancement of AMPAR activation.[55] Evidence that AMPAR potentiators possess antidepressant-like properties[56] suggests that a relative increase in AMPAR activation produces antidepressant efficacy. It is possible that our findings of increased expression of the AMPAR subunits in MDD indicate abnormal AMPAR regulation in MDD and that increased expression is a compensatory mechanism. In addition to abnormal expression of GluA subunits in MDD, our data also reveal increased expression of the KAR subunits, GluK1 and GluK2. Moreover, the strongest predictor of suicide in our cohort was GRIK3 (GluK3) expression in both sexes. KARs appear to regulate L-glutamate release by functioning as facilitatory or inhibitory autoreceptors during repetitive synaptic activation.[57,58] Although KAR activity can contribute to excitotoxic cell death,[57] the role of these receptors in the DLPFC of subjects with MDD still needs to be elucidated. Genetic variation of GRIK3 has been associated with recurrent MDD[20,59] and personality traits associated with MDD, such as anticipatory worry and harm avoidance.[18] Increased expression of GluK genes (GRIKs) in MDD could predict reduced glutamate release and perhaps increased severity of depressive symptoms.

In addition to the ionotropic GluRs, expression levels of some mGluRs were increased in MDD. Differential expression of mGluR1 (GRM1), mGluR4 (GRM4), mGluR5 (GRM5) and mGluR7 (GRM7) were detected in the DLPFC of subjects with MDD. All of these genes exhibited higher expression in females with MDD. Only GRM5 expression was significantly associated with MDD in the male groups (as discussed above). Recent studies show that microRNA regulation of mGluR4 is disrupted in male suicides, leading to relatively higher expression of mGluR4,[60] and therefore our findings in the female subjects may be consistent with these previous data. We also detected higher expression levels of the mGluR2 gene, GRM2, in the MDD-S relative to MDD-NS subjects. These effects were larger in the female subjects, but there was insufficient statistical power to draw sex-specific conclusions with respect to suicide as discussed earlier. In line with our findings, a previous postmortem study showed higher levels of mGluR2/3 binding in the PFC of male MDD subjects.[24] Antagonists for these receptors are likely to be beneficial in the treatment of anxiety and depression due to their ability to regulate presynaptic glutamate release.[37] MGluR2/3 antagonist drugs have been shown to have antidepressant efficacy in several animal models of depression.[61,62] Therefore, our data may indicate that mGluR2/3 antagonists would have efficacy in MDD patients with a high risk of suicide, if such patients could be identified *a priori*. GRM2 could also be a biomarker of suicide.

We have observed sex differences in glutamatergic gene expression when controls were compared with MDD patients. These sex differences may arise due to different risk alleles for MDD that are enriched in our female MDD patients. Alternatively, these differences in gene expression could arise due to hormonal differences between the sexes. Studies have shown that ovarian steroids can lead to increased expression of ionotropic GluRs and increased dendritic spine density in the cortex.[63] Repeated stress in male rats has been reported to be associated with reduced AMPAR and NMDAR expression and also reduced activity of these receptors. In contrast, in female rats exposed to stress, the expression of AMPAR and NMDARs was normalized through the activation of estrogen receptors, resulting in a neuroprotective and procognitive effect.[64,65] It is possible that, in our female patients, the activity of ovarian hormones is abnormal, leading to higher levels of glutamatergic gene expression and perhaps increased neuronal cell survival.

Although our data do not include analyses of estrogen levels in the female groups, analysis of gene expression in premenopausal and postmenopausal age groups (assuming an average menopausal age of 51 years), revealed significant differences in GRIN2C gene expression only. In rats, stress has been shown to increase the performance of females in memory tasks and to simultaneously increase anxiety.[66] We speculate that our data may indicate a molecular pathway that underpins the tendency for female patients to ruminate on past negative memories, which is positively correlated with depressive episodes.[67] Further analyses are required to elucidate the molecular mechanisms implicated by our data.

Studies of human postmortem brain are necessary to identify dysfunctions occurring at the molecular level in psychiatric disorders, but there are limitations to this approach, some of which are shared with all studies of human tissue. Limitations that are specific to investigations of postmortem brain include the lengthy times between death and brain removal, which may alter the quality of the RNA tested. Therefore, there were several covariates in our analyses, including measures of RNA integrity. Detailed toxicological analyses were also performed in this brain collection,[68] and these are helpful in distinguishing illness effects from medication effects in patients. From these measures, we know some postmortem subjects tested positive for the presence of antidepressant drugs, alcohol, nicotine, drugs of abuse and antipsychotic drugs. The gene expression differences that we have observed are unlikely to be a consequence of antidepressant treatment administered to the patients, because we detected no significant gene expression differences between the MDD patients who tested antidepressant positive compared with the patients who tested antidepressant negative in postmortem toxicological screens. The latter finding is not surprising given that no currently prescribed antidepressant therapy targets the glutamate system, although serotoninergic activity has been shown to alter glutamate release in cortical pyramidal neurons and therefore indirect effects are possible.[69] The relatively large size of the postmortem cohort tested meant that there was sufficient statistical power to detect a large effect in almost all analyses conducted. Only separate analyses of suicide in males and females lacked statistical power, but analyses of the total sample of MDD-S vs MDD-NS had sufficient statistical power to detect large effects. Therefore, some analyses of MDD-S relative to MDD-NS in the different sexes may have been falsely negative. Nevertheless, some effects, for example, the associations of GRIK3 with suicide, were large enough to be detected in both sexes in this cohort. We cannot rule out the possibility that an epiphenomenon is responsible for our findings, such as type, amount or duration of ante-mortem medication treatment. Replication in a second postmortem cohort is required to consolidate these findings.

In summary, we report association between increased DLPFC expression of several GluR genes and MDD, with the most striking increases in female MDD subjects and in the MDD subjects who died by suicide. Given that rapid antidepressant efficacy has been observed after treatment with the NMDAR antagonist drug ketamine, increased GluR expression in MDD and suicide should not be surprising. These data may indicate that specific abnormalities occur directly in the glutamatergic system in the DLPFC in MDD and that these abnormalities are most severe in female patients. Our findings may also reveal novel biomarkers for MDD and suicide. The mechanisms underlying the effects detected require further investigation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)