Differential expression of glutamate transporters and monoaminergic genes in major depression and suicide.

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Abstract

Accumulating evidence indicates that the glutamate and monoamine systems contribute to the pathophysiology of major depressive disorder (MDD) and suicide. We have tested the expression of genes encoding glutamate transporters and monoaminergic proteins in the dorsolateral prefrontal cortex (DLPFC) of MDD subjects who died by suicide (MDD-S, n=51), MDD non-suicide subjects (MDD-NS, n=28), and non-psychiatric controls (CTRL, n=32). We analyzed glutamate transporters (EAAT1, EAAT2, VGLUT1, and VGLUT2) and monoaminergic genes (SERT, NET, DAT, PMAT, VMAT, TPH1 and TPH2). Females but not males with MDD showed higher expression of all glutamate transporters relative to CTRLs ($P<0.05$). MDD-S groups of both sexes had higher VGLUT2 expression ($P<0.05$). MDD-S females who were antidepressant positive (+) had lower EAAT1 expression ($P=0.004$), perhaps indicating poor treatment response. Analyses of monoaminergic genes revealed lower VMAT1 expression ($P=0.002$) in MDD males, and conversely higher VMAT2 in MDD females ($P=0.004$). MDD females also had higher VMAT2, TPH2 and NET expression ($p<0.05$), and in contrast, MDD males had lower VMAT1 and PMAT expression. Therefore, we report sex differences in the expression of glutamate transporters and some monoaminergic genes in the DLPFC in MDD. Most of these findings are novel, but lower EAAT1 expression in MDD-S replicates previous studies. Lower EAAT1 expression coupled with higher VGLUT2 expression in MDD-S may lead to increased synaptic glutamate, neuronal loss and glial loss in the DLPFC in MDD and suicide reported previously. These deficits may contribute to lower DLPFC activity, poor problem solving and impaired executive function exhibited in severe depression and suicide.

Introduction

Major depressive disorder (MDD) is a chronic psychiatric disorder that leads to significant medical and economic burdens on individuals and societies. It affects mood and behavior, resulting in cognitive impairments, sleep disturbances, alteration of appetite, anxiety, and anhedonia. MDD is also characterized by thoughts of suicide. Over 50% of individuals who die by suicide have a primary diagnosis of MDD (1, 2).

Conventional antidepressant treatments target the monoamine system(3-6), but only one-third of patients demonstrate substantial improvement, and many fail to respond to treatment (7-9). The commonly prescribed antidepressant drugs include selective serotonin reuptake inhibitors (SSRIs), which are antagonists of the serotonin (5-Hydroxytryptamine, 5-HT) transporter protein (SERT); serotonin-norepinephrine reuptake inhibitors (SNRIs), which inhibit both SERT and the norepinephrine transporter protein (NET); tricyclic antidepressants (TCAs), which increase synaptic release of monoamines; and monoamine oxidase inhibitors (MAOIs), which inhibit the metabolism of monoamines (10). Although these treatments rapidly increase the synaptic levels of 5-HT, norepinephrine and dopamine, there is usually a delay of several weeks before patients achieve antidepressant efficacy. In contrast, glutamatergic drugs, such as ketamine, have rapid antidepressant efficacy (11) in treatment resistant, severely depressed patients, sometimes within hours of treatment (12, 13). These and other accumulating data indicate glutamatergic abnormalities (14-16) and differences between the sexes in depression.

MDD is sexually dimorphic, with two to three fold greater frequency (17, 18), and greater symptom severity in women (19, 20). These clinical observations may reflect sex differences in molecular mechanisms underlying MDD(21). Transcriptional abnormalities in cortico-limbic brain regions that are associated with MDD differ greatly between the sexes. When genes are abnormally expressed in MDD in both sexes, these are often altered in opposite directions (22).

Brain regions implicated in the pathophysiology of MDD and suicide are sexually dimorphic (23). Among these regions, the dorsolateral prefrontal cortex (DLPFC) is critical for optimal emotional and executive function (24). During emotional tasks, the DLPFC is deactivated (25-27). Brain imaging studies show that executive function is disrupted in MDD patients (28) due to impaired DLPFC activity (29), perhaps due to reduced numbers of DLPFC synapses in MDD (30). Modulating DLPFC activity (31-36) may be necessary for antidepressant efficacy. The rapid antidepressant effects of ketamine, an antagonist of the N-methyl-D-aspartate receptor (NMDAR), coincide with rapid increases in spine synapses in the prefrontal cortex and activation of downstream signaling pathways that stimulate synapse formation (37). Accumulating data, including from our laboratory, indicate altered glutamatergic gene expression in the DLPFC in MDD (38-40).

There is a well-documented association between MDD and disrupted transmission within the glutamate(14-16) and monoamine systems (41-46). Although many of the conventional antidepressants target monoaminergic transmission, there are few studies reporting mRNA expression levels of monoaminergic genes in the DLPFC in MDD. In the current study, we have tested the hypothesis that glutamate transporter and monoaminergic gene expression are abnormal in the DLPFC of MDD patients, and that these gene expression abnormalities differ between the sexes. We have tested this hypothesis in a large cohort of postmortem subjects from three diagnostic groups: MDD patients who died by suicide (MDD-S), MDD patients who did not die by suicide (MDD-NS), and a comparison group of control subjects with no history of psychiatric illness (CTRL). Given that antidepressant treatment response may be predicted by suicidality in patients (47), we also tested if gene expression differs between the MDD-S and MDD-NS groups when assessing only subjects who are likely to have been compliant with antidepressant therapy at their time of death, as indicated by a positive test for antidepressants postmortem (MDD-S+ and MDD-NS+). Lastly, we have tested if any altered monoaminergic gene expression in the DLPFC results from antidepressant treatment, by comparing MDD patients who either did or did

not test positive for antidepressants postmortem (MDD+ and MDD-). The results of this study indicate that glutamate transporter expression was upregulated in female but not male subjects with MDD relative to CTRL groups. We also report sex-specific effects in the expression of some monoaminergic genes.

Materials and Methods

Subjects

Human postmortem brain specimens were obtained from the Brain Collection of the Clinical Brain Disorders Branch (subsequently renamed Human Brain Collection Core) at the National Institute of Mental Health (NIMH). Brains were collected from the Offices of the Chief Medical Examiner in Washington, DC and in Northern Virginia, USA. Postmortem interval (PMI) was calculated as time elapsed between death and tissue freezing, in hours. Frozen tissue from the DLPFC was obtained from two groups of subjects: (1) patients diagnosed with MDD, based on Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria; and (2) a control group with no history of substance abuse, psychiatric or neurological disorders. Each case had a 20-item telephone screening performed by a physician on the day of donation to document 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, incorporating all of the above information. Two board-certified psychiatrists reviewed these cases to arrive at lifetime DSM-IV diagnoses. **Table 1** summarizes the demographic variables of the postmortem subjects.

The medical examiner's office performed toxicological screenings in the blood, brain or other available matrix for prescribed medications, drugs of abuse and other substances that may have been related to the cause of death. Supplemental toxicology screening was done by the NIMH in order to fill in any gaps in testing for both prescription medications and drugs of abuse. Selective supplemental toxicology was performed depending upon the history of each patient. To measure the pH of the brain, 100 mg of homogenized frozen brain tissue was mixed with 1ml 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 of death, to exclude cases with hepatic or renal disease (which may cause increases in astrogia). Macroscopic and

microscopic examination of the brain, 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 (48).

Preparation of cDNA

Laboratory personnel were blind to clinical data during the experiments. RNA was extracted from gray matter and complementary DNA (cDNA) prepared by standard methods (39). RNA integrity number (RIN), was measured using an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). The average RIN value was 8.3, which indicates high quality RNA (**Supplementary Table 1**). Equal quantities of RNA from each subject were used to synthesize cDNA 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 to generate a calibration curve according to the relative standard curve method (<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 μ l pooled assays and 44 μ l TaqMan JumpStart (Sigma-Aldrich, St Louis, MO, USA), was preamplified for 14 cycles as previously described (49, 50).

Gene expression assays

We measured gene expression as previously described (39). Assays are summarized in **Supplementary Table 2**. Quantitative PCR (QPCR) assays were performed in duplicate using the Applied Biosystems ViiA 7 System (Life Technologies, Grand Island, NY, USA).

Housekeeping genes used to normalize the gene expression data were eliminated if associated with independent variables and covariates in analyses of variance (ANOVA). Of six housekeeping (HK) genes tested, three (GUSB, B2M, ACTB) were included in our analyses. The geometric mean of HK gene expression was not associated with diagnosis or sex, nor was there an interaction of sex by diagnosis. Normalizing data with multiple HK 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 tested the hypothesis that dysfunction of glutamate and monoamine systems occurs in MDD and suicide by examining the expression of glutamate transporter genes and monoaminergic genes in the DLPFC of a postmortem cohort including patients with MDD (n=80) and a control group of psychiatrically healthy subjects (n=32). We used multivariate analysis of covariance (MANCOVA) to investigate main effects, and performed *posthoc* analyses of individual genes using univariate ANCOVA, with PMI, age at death, brain pH, and RIN included as covariates. We used Box's M test to confirm that the observed covariance matrices for the dependent variables was equal across groups. We used Levene's test of homogeneity of variance to confirm that the variance in each group compared was similar. If data that violated Levene's test, we used the non-parametric Mann-Whitney U test for data analysis. We also tested the relationship between gene expression and demographic variables using analysis of variance (**Supplementary Table 1**). We calculated the false discovery rate (FDR) to correct for the effects of multiple comparisons (51). Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 24.0. (IBM Corp., Armonk, NY, USA).

In light of recent reports, including our own, indicating dramatic sexual dimorphism in gene expression profiles in MDD (21, 22, 39), we analyzed males and females separately.

For glutamate transporter gene expression analyses, the following model was tested using MANCOVA:

$$1. \text{ Gene expression (y)} = \beta_0 + \beta_1\text{PMI} + \beta_2\text{Age} + \beta_3\text{pH} + \beta_4\text{RIN} + \beta_5\text{Diagnosis}$$

Diagnosis was a two-level factor, Control vs MDD.

Subsequently, we compared subgroups of the MDD cases to test for potential predictors of suicide, using the following model:

$$2. \text{ Gene expression (y)} = \beta_0 + \beta_1\text{PMI} + \beta_2\text{Age} + \beta_3\text{pH} + \beta_4\text{RIN} + \beta_5\text{Suicide}$$

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

We then compared subgroups of the MDD patients who tested positive for antidepressants postmortem, and either did or did not die by suicide, using the model below:

$$3. \text{ Gene expression (y)} = \beta_0 + \beta_1\text{PMI} + \beta_2\text{Age} + \beta_3\text{pH} + \beta_4\text{RIN} + \beta_5\text{Suicide}$$

Suicide was a two-level factor, MDD-NS+ vs MDD-S+.

For monoamine gene expression analysis, the following model was tested using MANCOVA:

$$4. \text{ Gene expression (y)} = \beta_0 + \beta_1\text{PMI} + \beta_2\text{Age} + \beta_3\text{pH} + \beta_4\text{RIN} + \beta_5\text{Diagnosis}$$

Diagnosis was a two-level factor, Control vs MDD.

We then compared subgroups of the MDD cases to test for potential predictors of suicide, using the following model:

$$5. \text{ Gene expression (y)} = \beta_0 + \beta_1\text{PMI} + \beta_2\text{Age} + \beta_3\text{pH} + \beta_4\text{RIN} + \beta_5\text{Suicide}$$

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

We then compared subgroups of the MDD patients who tested positive for antidepressants postmortem, and either did or did not die by suicide, using the model below:

6. Gene expression (y) = $\beta_0 + \beta_1\text{PMI} + \beta_2\text{Age} + \beta_3\text{pH} + \beta_4\text{RIN} + \beta_5\text{Suicide}$

Suicide was a two-level factor, MDD-S+ vs MDD-NS+.

Lastly, we compared monoaminergic gene expression between MDD patients who either did or did not test positive for antidepressants postmortem, using the following model:

7. Gene expression (y) = $\beta_0 + \beta_1\text{PMI} + \beta_2\text{Age} + \beta_3\text{pH} + \beta_4\text{RIN} + \beta_5\text{Antidepressant}$

Antidepressant was a two-level factor, MDD+ vs MDD-.

Results

Our multivariate analyses revealed increased expression of glutamate transporters in female patients diagnosed with MDD relative to controls ($F_{4,45} = 2.77$, $P = 0.038$). *Posthoc* univariate analyses indicated that EAAT2, VGLUT1, and VGLUT2 genes exhibited significantly increased expression in MDD subjects relative to controls (**Figure 1A, Table 1A**). In contrast, we observed no significant differences in glutamatergic gene expression in the male groups.

We also sought to determine potential predictors of suicide risk by analyzing gene expression in MDD suicides (MDD-S) compared to MDD non-suicides (MDD-NS). In female subjects, multivariate analysis revealed a significant difference in glutamate transporter gene expression between MDD-S and MDD-NS groups ($F_{4,31} = 3.87$, $P = 0.012$). *Posthoc* univariate analyses (**Figure 1B, Table 1A**) showed lower expression of EAAT1 and higher expression of VGLUT2 in the female MDD-S group compared to the female MDD-NS group ($F_{1,76} = 4.60$, $P = 0.035$). There were no significant differences in glutamate transporter expression between the male MDD-S and MDD-NS subjects.

An additional analysis of potential predictors of treatment response included only MDD patients who tested positive for antidepressants postmortem. In female subjects, multivariate analysis showed a significant difference in glutamate transporter gene expression between MDD-S+ and MDD-NS+ groups ($F_{4,11} = 5.07$, $P = 0.015$). *Posthoc* univariate analyses revealed lower expression levels of EAAT1 in the female MDD-S+ group compared to the female MDD-NS+ group (**Figure 1C, Table 1B**). In male subjects, multivariate analyses of glutamate transporter expression showed no differences between the MDD-S+ and MDD-NS+ groups. However, *post-hoc* univariate analyses indicated increased expression of VGLUT2 in the MDD-S+ group compared to the MDD-NS+ group.

Subsequent analyses focused on the expression of monoaminergic genes. Multivariate analyses showed a significant effect of diagnosis in female subjects ($F_{8,41} = 2.41$, $P = 0.031$) and male subjects ($F_{8,45} = 2.23$, $P = 0.043$). *Post-hoc* univariate analyses revealed increased expression of

VMAT2, NET, and TPH2 in females with MDD relative to controls (**Figure 2A, Table 2**). In contrast, univariate analyses indicate that males with MDD have decreased expression of VMAT1 and decreased expression of PMAT relative to controls (**Figure 2B, Table 2**).

Further analyses of monoaminergic gene expression revealed no significant differences between MDD-S and MDD-NS groups in either males or females (**Supplementary Figure 1A, Supplementary Figure 1B, and Supplementary Table 3**) regardless of antidepressant positive status (**Supplementary Figure 1C, Supplementary Figure 1D, Supplementary Table 4**). Moreover, analyses revealed no differences in monoaminergic gene expression when we compared MDD+ with MDD- patients in groups of either sex (**Supplementary Figure 1E, Supplementary Figure 1F, and Supplementary Table 5**).

Some of the data that we report here replicates previous studies, as outlined in the Discussion. When we correct these analyses for false discovery using the method proposed by Benjamini and Hochberg (51), these findings do not remain statistically significant. However, we note that 12 of the 88 *post-hoc* tests (14%) show an uncorrected p value ≤ 0.05 , which is approximately three times the rate of positives expected with an α (false positive) threshold of 5%.

Discussion

Our findings support the hypothesis that both glutamatergic and monoaminergic genes have abnormal expression in the DLPFC in MDD and suicide. We observed sex differences in these gene expression measures when comparing MDD with CTRLs, and when we compared MDD-S with MDD-NS groups.

Sex differences in glutamate transporter expression in MDD.

MDD females but not MDD males had higher expression levels of glutamate transporter genes compared with CTRLs of the same sex. These data add to the accumulating evidence that there are glutamatergic abnormalities in MDD and suicide, particularly in females (39). Upregulation of VGLUTs may lead to the elevated levels of synaptic or extracellular glutamate observed in the DLPFC in MDD (52). Elevated glutamate levels may result from increased activity at the glutamate synapse in MDD females. These findings are consistent with our previous report of elevated glutamate receptor expression in MDD females(39). In females, estrogen activates estrogen receptors (ER), which bind to estrogen response elements (EREs) upstream of the promoters of specific genes in the prefrontal cortex (53). EREs are located immediately upstream of the EAAT2 gene (54) therefore upregulated EAAT2 expression may result from elevated estrogen signaling during stress. The increased expression of EAAT proteins in MDD females may be a compensatory mechanism by which excess glutamate is removed from the synapse during stressful life events (55, 56).

Previous studies of mixed-sex cohorts, including mostly male subjects, show decreased expression of glutamate transporters in MDD in the frontal cortex (38, 57-60). In contrast, we detected upregulated expression of glutamate transporters in the DLPFC of MDD females but not in MDD males. However, increased expression of synapse-related genes in the DLPFC of MDD females has been reported previously (22).

Glutamate transporter expression is abnormal in depressed suicides

Sex differences in gene expression in MDD may underpin the sex differences in symptoms and severity of MDD (21, 22). Completed suicide may be an indicator of more severe illness in MDD. Male and female MDD-S groups showed similar abnormalities of glutamate transporter expression relative to same sex MDD-NS groups, although we detected larger differences in the females. Increased expression of VGLUT2 in MDD-S subjects, may predict increased release of glutamate at the synapse. In addition, EAAT1 expression was relatively lower in MDD-S patients. Other studies of mixed-sex cohorts have reported decreased expression of EAAT1 and EAAT2 in the DLPFC in MDD-S(58), and in suicides with schizophrenia (61) with smaller effects in males. These data are also consistent with a recent study of mostly male subjects (40). Moreover, previous reports indicate lower levels of EAAT1 and EAAT2 expression in MDD in the frontal cortex (38, 57-59), the hippocampus (62) and locus coeruleus (63). In combination, these data indicate lower EAAT expression in the DLPFC is associated with severe depression, in both sexes.

Expression of EAAT1 is primarily in the astrocyte in the DLPFC (55, 56, 64) and therefore, lower EAAT1 expression may indicate a relative loss of glia or glial function in the DLPFC of the MDD-S females. Lower EAAT activity would reduce the efficiency of removal of extracellular glutamate. **Figure 3** summarizes the functions of the glutamate transporters and their relative roles in regulating synaptic glutamate levels. In combination, if MDD-S groups have reduced EAAT1 expression and increased VGLUT2 expression, excess extracellular and synaptic glutamate could result, leading to excitotoxic cell death in the DLPFC (65, 66). If validated, these findings could explain the reduced DLPFC activity observed in suicidal patients. Reduced activity in the DLPFC would lead to poor problem solving and poor planning (67), which may result in suicide attempts in response to stress.

Sex differences in glutamate transporter expression in antidepressant treatment responders

We have considered suicide completion in antidepressant positive MDD patients as an indication of poor response to antidepressant treatment. We compared the 'poor responder group' (MDD-S+) with a group of MDD patients who were antidepressant positive but who did not die by suicide i.e. 'good responders' (MDD-NS+). In females, we detected lower EAAT1 expression in MDD-S+ but in male MDD-S+, we detected higher VGLUT2 expression relative to MDD-NS+ of the same sex. Reduced EAAT1 or increased VGLUT2 would lead to higher glutamate levels in the synapse of both sexes (65). One previous report showed higher levels of synaptic glutamate in the DLPFC of MDD patients with poor treatment response (68). Therefore, males and females who respond poorly to antidepressants may have similarly elevated glutamate levels in the DLPFC but by slightly different mechanisms i.e. males may have greater glutamate release from the presynaptic neuron while females may have less efficient reuptake of extracellular glutamate. Therefore, our data indicate that different molecular or cellular abnormalities in males and females may underlie this abnormality of glutamate system regulation. One previous report indicates that female rats have enhanced sensitivity to ketamine during proestrus stage of the menstrual cycle, which is mediated by estradiol activation of the estrogen receptors, leading to greater activation of synaptic plasticity related kinases within prefrontal cortex (69).

Sex differences in monoamine transporter expression in depression

Our expression analyses of monoaminergic genes included genes encoding the monoamine transporters and two isoenzymes of tryptophan hydroxylase. Considerable evidence implicates these genes in the pathophysiology of MDD and suicide (70) and several are targets of antidepressant drugs(71). We detected lower expression of VMAT1 and PMAT in MDD males compared with male controls. In the presynaptic terminals of monoaminergic neurons, vesicular monoamine transporters (VMATs) sequester 5-HT, dopamine, and norepinephrine into synaptic vesicles for release, which may be reduced if VMAT1 expression is lower in MDD males. PMAT is membrane bound and has a strong kinetic preference for 5-HT and dopamine although PMAT

also transports other mono-amines, such as histamine, norepinephrine, and epinephrine(72). Lower PMAT expression in MDD males could indicate that there is reduced transport of several monoamines from the synapse. These altered expression levels are unlikely to be a result of antidepressant treatment received by the MDD patients, because we observed no differences in the expression of any monoaminergic gene between the antidepressant positive and antidepressant negative MDD patients.

In contrast with the MDD males, we observed increased expression of VMAT2, NET, and TPH2, in MDD females relative to controls. Comparison of antidepressant positive and antidepressant negative MDD female subjects indicates that the monoaminergic gene expression differences that we observe in the females are unlikely to be a result of antidepressant treatment. It is possible that some of these molecular abnormalities may predict antidepressant efficacy. Women show better response to SSRIs and MAOIs, and men have better response to TCAs (22). SSRIs increase synaptic 5-HT levels, while MAOIs are non-specific inhibitors of monoamine metabolism(10). The current data indicate disruption of gene expression in 5-HT neurons (due to TPH2 expression) and norepinephrine neurons (due to NET expression) in the DLPFC in women and perhaps drugs that target these systems, such as SSRIs and SNRIs will have greater antidepressant efficacy in females with MDD. In males, altered VMAT1 and PMAT expression indicates abnormalities that may not occur in any specific monoaminergic neuron(72) in MDD, which may be why non-specific monoaminergic drugs such as TCAs will have greater efficacy in males. While we observed no variation of DAT expression between our diagnostic groups, we cannot rule out abnormal dopaminergic transmission in MDD because we have observed altered expression of non-specific transporters such as the VMATs and PMAT in MDD. Moreover, VGLUT2, which was elevated in the suicide groups, is expressed in both glutamate and dopamine neurons (73). The upregulation of NET and TPH2 in MDD females may be due to stress-induced stimulation of estrogen response elements (EREs) immediately upstream of the NET, and TPH2 genes (54).

Our data may be consistent with a report of increased TPH2 mRNA expression in the prefrontal cortex in suicide (74), although there have been conflicting data (75). The expression of these genes was not associated with antidepressant positive status, indicating that these findings may not be influenced by the medication administered to these patients. We observed no abnormalities of SERT expression in MDD, which is consistent with a meta-analysis of five postmortem studies of the prefrontal cortex (76). To our knowledge, we are the first to report abnormal expression of the other VMAT, PMAT and NET in the DLPFC in MDD. We did not observe differences in monoaminergic gene expression in any other analysis.

Limitations of this study include those that are common to all postmortem analyses of human brain, but investigations of human postmortem brain are necessary for detailed cellular and molecular studies of neurological disorders. Demographic variables (**Supplementary Table 1**) including RIN values, sample number and detailed toxicological analyses performed are the strengths of this study (77). We based this study on strong neurobiological hypotheses, which is probably why we report three times the number of positive findings than would be expected by chance alone. Nevertheless, when we corrected our data for false discovery, the findings are no longer significant. Sole reliance on probability values to determine significance may be erroneous (78), and replication is a more reliable indicator of true discoveries. Replication is unusual when investigating human subjects, but in spite of this, some data that we report here replicate previous studies. The novel findings that we report will require replication to separate the true positives from the false positive data. In addition, mRNA abundance does not always correlate with protein activity. If some of the mRNA measured included non-coding RNAs, then altered mRNA levels would be less likely to correlate directly with protein activity.

Therefore, with the limitations we note in mind, we report sex differences in the expression of glutamate transporters and some monoaminergic genes in the DLPFC in MDD. Most of these findings are novel, but lower EAAT1 expression in MDD-S replicates previous studies. It should be noted that replication is rare in studies of human subjects. Lower EAAT1 expression coupled

with higher VGLUT2 expression in MDD-S may lead to increased synaptic glutamate, neuronal loss and glial loss in the DLPFC in MDD and suicide reported previously. These deficits may contribute to lower DLPFC activity, poor problem solving and impaired executive function exhibited in severe depression and suicide. Taken together, these data highlight the importance of considering sex when investigating the biological basis of MDD and suicide. Future analyses of additional regions within the cortico-limbic circuitry in MDD would further elucidate the pathophysiological mechanisms of depression and suicide (79, 80).

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Table 1. Expression of glutamate transporter genes in MDD and suicide.

All analyses conducted in the DLPFC.

Gene	Group	Sex	N	Mean	s.d.		Mean diff	Test statistic	p-value
EAAT1	CTRL	M	19	1.18	0.34	M	0.178	$F_{1,52} = 2.93$	$p = 0.093$
		F	13	1.11	0.38	F	-0.203	$F_{1,48} = 2.97$	$p = 0.091$
	MDD	M	39	1.20	0.43				
		F	41	1.33	0.41				
EAAT2	CTRL	M	19	1.23	0.33	M	0.141	$F_{1,52} = 1.62$	$p = 0.209$
		F	13	1.05	0.40	F	-0.385	$F_{1,48} = 6.57$	$p = 0.014$
	MDD	M	39	1.29	0.46				
		F	41	1.45	0.48				
VGLUT1	CTRL	M	19	1.47	0.52	M	0.058	$F_{1,52} = 0.24$	$p = 0.626$
		F	13	1.12	0.49	F	-0.290	$F_{1,48} = 4.90$	$p = 0.032$
	MDD	M	39	1.46	0.30				
		F	41	1.47	0.35				
VGLUT2	CTRL	M	19	0.94	0.43	M	0.060	$U = 338^{\dagger}$	$p = 0.590$
		F	13	0.60	0.35	F	-0.214	$F_{1,48} = 6.85$	$p = 0.012$
	MDD	M	39	0.88	0.20				
		F	41	0.84	0.22				
EAAT1	MDD-NS	M	15	1.32	0.54	M	0.105	$F_{1,33} = 0.80$	$p = 0.378$
		F	13	1.52	0.32	F	0.242	$F_{1,34} = 4.31$	$p = 0.046$
	MDD-S	M	24	1.13	0.34				
		F	27	1.24	0.43				
EAAT2	MDD-NS	M	15	1.44	0.61	M	0.149	$F_{1,33} = 1.41$	$p = 0.243$
		F	13	1.58	0.35	F	0.120	$F_{1,34} = 0.60$	$p = 0.445$
	MDD-S	M	24	1.20	0.32				
		F	27	1.39	0.54				
VGLUT1	MDD-NS	M	15	1.41	0.22	M	-0.092	$F_{1,33} = 0.82$	$p = 0.371$
		F	13	1.42	0.28	F	-0.190	$F_{1,34} = 2.19$	$p = 0.148$
	MDD-S	M	24	1.49	0.34				
		F	27	1.51	0.37				
VGLUT2	MDD-NS	M	15	0.82	0.17	M	-0.085	$F_{1,33} = 1.78$	$p = 0.192$
		F	13	0.77	0.16	F	-0.155	$F_{1,34} = 4.21$	$p = 0.048$
	MDD-S	M	24	0.92	0.20				
		F	27	0.90	0.22				
EAAT1	MDD-NS+	M	6	1.10	0.61	M	0.019	$F_{1,13} = 0.00$	$p = 0.946$
		F	5	1.61	0.36	F	0.553	$F_{1,14} = 11.88$	$p = 0.004$
	MDD-S+	M	13	1.23	0.34				
		F	15	1.19	0.39				
EAAT2	MDD-NS+	M	6	1.10	0.63	M	0.013	$F_{1,13} = 0.00$	$p = 0.961$
		F	5	1.66	0.30	F	0.426	$F_{1,14} = 2.81$	$p = 0.116$
	MDD-S+	M	13	1.27	0.27				
		F	15	1.32	0.50				
VGLUT1	MDD-NS+	M	6	1.49	0.17	M	-0.245	$F_{1,13} = 3.73$	$p = 0.075$
		F	5	1.55	0.37	F	-0.141	$F_{1,14} = 0.36$	$p = 0.555$
	MDD-S+	M	13	1.55	0.25				
		F	15	1.52	0.32				
VGLUT2	MDD-NS+	M	6	0.84	0.14	M	-0.203	$F_{1,13} = 4.71$	$p = 0.049$
		F	5	0.80	0.22	F	-0.168	$F_{1,14} = 1.33$	$p = 0.267$
	MDD-S+	M	13	0.93	0.19				
		F	15	0.91	0.22				

Abbreviations: Control (CTRL), standard deviation (sd), Major Depressive Disorder (MDD), number of subjects (N), non-suicide (NS), suicide (S), probability (p), antidepressant positive at time of death (+). Gene names listed in Supplementary Table 2.

Table 2. Sex differences in monoamine gene expression in depression. Depressed males had lower expression of VMAT1 relative to male controls, while depressed females had higher expression of VMAT2, NET and TPH2 relative to female controls. All analyses conducted in the DLPFC.

Gene	Group	Sex	N	Mean	s.d.	Mean diff	statistic	p-value
VMAT1	CTRL	M	19	0.88	0.71	M 0.586	$F_{1,52} = 10.62$	$p = 0.002$
		F	13	0.34	0.71	F -0.263	$F_{1,48} = 1.59$	$p = 0.214$
	MDD	M	39	0.46	0.58			
		F	41	0.64	0.63			
VMAT2	CTRL	M	19	1.33	0.73	M 0.211	$F_{1,52} = 0.97$	$p = 0.329$
		F	13	0.94	0.56	F -0.673	$F_{1,48} = 8.92$	$p = 0.004$
	MDD	M	39	1.17	0.67			
		F	41	1.43	0.75			
PMAT	CTRL	M	19	1.28	0.44	M 0.194	$F_{1,52} = 3.97$	$p = 0.050$
		F	13	1.15	0.66	F -0.250	$F_{1,48} = 2.86$	$p = 0.097$
	MDD	M	39	1.11	0.30			
		F	41	1.33	0.44			
DAT	CTRL	M	19	1.10	1.29	M 0.445	$F_{1,52} = 0.89$	$p = 0.351$
		F	13	0.51	0.80	F -0.635	$U = 184^{\dagger}$	$p = 0.088$
	MDD	M	39	1.22	1.85			
		F	41	1.65	2.40			
NET	CTRL	M	19	0.79	0.38	M -0.033	$F_{1,52} = 0.04$	$p = 0.834$
		F	13	0.50	0.43	F -0.325	$F_{1,48} = 6.32$	$p = 0.015$
	MDD	M	39	0.78	0.55			
		F	41	0.74	0.44			
SERT	CTRL	M	19	0.93	0.67	M 0.220	$F_{1,52} = 2.55$	$p = 0.117$
		F	13	1.76	3.55	F 0.659	$F_{1,48} = 1.23$	$p = 0.272$
	MDD	M	39	0.73	0.33			
		F	41	0.92	0.79			
TPH1	CTRL	M	19	0.86	0.33	M -0.046	$F_{1,52} = 0.17$	$p = 0.678$
		F	13	0.97	0.91	F -0.029	$F_{1,48} = 0.03$	$p = 0.867$
	MDD	M	39	0.92	0.37			
		F	41	0.96	0.37			
TPH2	CTRL	M	19	0.90	0.23	M 0.007	$F_{1,52} = 0.01$	$p = 0.919$
		F	13	0.63	0.40			
	MDD	M	39	0.87	0.25	F -0.307	$U = 144^{\dagger}$	$p = 0.013$
		F	41	0.93	0.21			

Abbreviations: Control (CTRL), standard deviation (sd), Major Depressive Disorder (MDD), number of subjects (N), probability (p). [†]Mann-Whitney U Test. Gene names listed in Supplementary Table 1.

Supplementary Table 1. Demographics of postmortem subjects.

Diagnosis	Sex	N	Suicide (Y / N)	Antidepressant (+ / -)	Age (mean $\pm$ sd)	PMI (mean $\pm$ sd)	brain pH (mean $\pm$ sd)	RIN (mean $\pm$ sd)
CTRL	M	19	0 / 19	0 / 19	39.4 $\pm$ 12.7	30.6 $\pm$ 13.4	6.6 $\pm$ 0.3	8.3 $\pm$ 0.5
	F	13	0 / 13	0 / 13	38.9 $\pm$ 16.2	26.3 $\pm$ 12.0	6.4 $\pm$ 0.3	8.0 $\pm$ 1.1
MDD	M	39	15 / 24	19 / 7	41.9 $\pm$ 14.3	35.6 $\pm$ 27.3	6.4 $\pm$ 0.2	8.1 $\pm$ 0.8
	F	41	13 / 27	20 / 8	44.0 $\pm$ 14.8	35.8 $\pm$ 17.3	6.2 $\pm$ 0.3	8.1 $\pm$ 0.7

Abbreviations: Age at death in years (Age), antidepressant positive at time of death (+), antidepressant negative at time of death (-), Female (F), Male (M), number of subjects (N), Postmortem interval in hours (PMI), RNA integrity number (RIN), standard deviation (sd).

Supplementary Table 2. Gene expression assays tested in postmortem tissue.

Gene	Protein	Assay
SLC1A2	Excitotoxic Amino Acid Transporter 2, EAAT2	Hs001102423_m1
SLC1A3	Excitatory Amino Acid Transporter 1, EAAT1	Hs00188193_m1
SLC17A6	Vesicular Glutamate Transporter 2, VGLUT2	Hs00220439_m1
SLC17A7	Vesicular Glutamate Transporter 1, VGLUT1	Hs00220404_m1
SLC6A2	Norepinephrine Transporter, NET	Hs00426573_m1
SLC6A3	Dopamine Transporter, DAT	Hs00997364_m1
SLC6A4	Serotonin Transporter, SERT	Hs00984349_m1
SLC29A4	Plasma Membrane Monoamine Transporter, PMAT	Hs00928283_m1
SLC18A1	Vesicular Amine Transporter 1, VMAT1	Hs00915193_m1
SLC18A2	Vesicular Amine Transporter 2, VMAT2	Hs00996835_m1
TPH1	Tryptophan hydroxylase 1	Hs00188220_m1
TPH2	Tryptophan hydroxylase 2	Hs00542783_m1
ACTB	Beta-actin	Hs9999903_m1
B2M	Beta-2-microglobulin	Hs9999907_m1
GUSB	Beta-glucuronidase	Hs9999908_m1

Supplementary Table 3. Monoamine gene expression in the DLPFC does not differ between depressed suicides and non-suicides.

Gene	Group	Sex	N	Mean	s.d.	Mean diff	statistic	p-value
VMAT1	MDD-NS	M	15	0.61	0.73	M 0.195	U = 157 [†]	p = 0.521
		F	13	0.72	0.61	F 0.076	F _{1,34} = 0.10	p = 0.758
	MDD-S	M	24	0.37	0.45			
		F	27	0.61	0.66			
VMAT2	MDD-NS	M	15	1.10	0.76	M -0.123	F _{1,33} = 0.27	p = 0.608
		F	13	1.35	0.65	F 0.008	F _{1,34} = 0.00	p = 0.975
	MDD-S	M	24	1.21	0.62			
		F	27	1.47	0.65			
PMAT	MDD-NS	M	15	1.13	0.28	M 0.041	F _{1,33} = 0.17	p = 0.682
		F	13	1.25	0.32	F -0.128	F _{1,34} = 0.66	p = 0.422
	MDD-S	M	24	1.09	0.31			
		F	27	1.39	0.32			
DAT	MDD-NS	M	15	1.26	2.32	M -0.284	F _{1,33} = 0.25	p = 0.621
		F	13	1.76	2.56	F -0.237	F _{1,34} = 0.07	p = 0.788
	MDD-S	M	24	1.19	1.54			
		F	27	1.66	2.40			
NET	MDD-NS	M	15	0.85	0.60	M 0.209	F _{1,33} = 1.34	p = 0.255
		F	13	0.73	0.21	F 0.082	U = 171 [†]	p = 0.897
	MDD-S	M	24	0.73	0.52			
		F	27	0.76	0.53			
SERT	MDD-NS	M	15	0.68	0.28	M -0.103	F _{1,33} = 0.90	p = 0.348
		F	13	0.92	0.25	F 0.107	F _{1,34} = 0.12	p = 0.727
	MDD-S	M	24	0.76	0.35			
		F	27	0.94	0.96			
TPH1	MDD-NS	M	15	0.79	0.36	M -0.207	F _{1,33} = 2.78	p = 0.105
		F	13	0.95	0.30	F -0.007	F _{1,34} = 0.00	p = 0.964
	MDD-S	M	24	1.00	0.37			
		F	27	0.97	0.41			
TPH2	MDD-NS	M	15	0.81	0.15	M -0.062	U = 148 [†]	p = 0.368
		F	13	0.90	0.21	F -0.043	F _{1,34} = 0.35	p = 0.558
	MDD-S	M	24	0.90	0.30			
		F	27	0.96	0.20			

Abbreviations: Antidepressant positive at time of death (+), Control (CTRL), standard deviation (sd), Major Depressive Disorder (MDD), number of subjects (N), probability (p). Female (F), Male (M), Major Depressive Disorder Non-Suicide (MDD-NS), Major Depressive Disorder Suicide (MDD-S), [†]Mann-Whitney U Test. Gene names listed in Supplementary Table 1.

[†]Mann-Whitney U Test, Gene names are listed in Supplementary Table 1.

Supplementary Table 4. Monoamine gene expression in the DLPFC does not differ between MDD-S and MDD-NS who were antidepressant positive at time of death by suicide.

Gene	Group	Sex	N	Mean	s.d.		Mean diff	statistic	p-value
VMAT1	MDD-NS+	M	6	0.19	0.41	M	-0.278	$F_{1,13} = 2.39$	$p = 0.146$
		F	5	0.81	0.83	F	0.108	$F_{1,14} = 0.05$	$p = 0.829$
	MDD-S+	M	13	0.21	0.30				
		F	15	0.52	0.66				
VMAT2	MDD-NS+	M	6	0.81	0.88	M	-0.167	$F_{1,13} = 0.17$	$p = 0.688$
		F	5	1.08	0.61	F	0.251	$F_{1,14} = 0.30$	$p = 0.595$
	MDD-S+	M	13	1.21	0.55				
		F	15	1.38	0.66				
PMAT	MDD-NS+	M	6	1.26	0.35	M	0.263	$F_{1,13} = 2.55$	$p = 0.134$
		F	5	1.28	0.38	F	0.218	$F_{1,14} = 0.54$	$p = 0.474$
	MDD-S+	M	13	1.05	0.23				
		F	15	1.44	0.52				
DAT	MDD-NS+	M	6	0.17	0.42	M	-1.455	$U = 17^{\dagger}$	$p = 0.058$
		F	5	2.97	3.80	F	0.502	$F_{1,14} = 0.04$	$p = 0.841$
	MDD-S+	M	13	1.63	1.86				
		F	15	2.31	2.90				
NET	MDD-NS+	M	6	1.09	0.74	M	0.654	$F_{1,13} = 4.62$	$p = 0.051$
		F	5	0.68	0.20	F	0.178	$F_{1,14} = 0.27$	$p = 0.608$
	MDD-S+	M	13	0.64	0.41				
		F	15	0.75	0.52				
SERT	MDD-NS+	M	6	0.62	0.33	M	0.135	$F_{1,13} = 0.52$	$p = 0.483$
		F	5	0.97	0.31	F	0.322	$F_{1,14} = 1.32$	$p = 0.269$
	MDD-S+	M	13	0.68	0.32				
		F	15	0.74	0.45				
TPH1	MDD-NS+	M	6	0.78	0.24	M	-0.227	$F_{1,13} = 1.11$	$p = 0.312$
		F	5	0.93	0.23	F	-0.079	$F_{1,14} = 0.08$	$p = 0.781$
	MDD-S+	M	13	1.05	0.34				
		F	15	0.92	0.38				
TPH2	MDD-NS+	M	6	0.89	0.84	M	0.031	$F_{1,13} = 0.06$	$p = 0.805$
		F	5	0.85	0.20	F	-0.144	$F_{1,14} = 2.16$	$p = 0.164$
	MDD-S+	M	13	0.88	0.21				
		F	15	0.98	0.18				

Abbreviations: Antidepressant positive at time of death (+), Control (CTRL), standard deviation (sd), Major Depressive Disorder (MDD), number of subjects (N), probability (p). Female (F), Male (M), Major Depressive Disorder Non-Suicide (MDD-NS), Major Depressive Disorder Suicide (MDD-S), [†]Mann-Whitney U Test. Gene names listed in Supplementary Table 1.

Supplementary Table 5. Monoamine gene expression in the DLPFC does not differ between depressed subjects who were antidepressant positive and antidepressant negative.

Gene	Group	Sex	N	Mean	s.d.	Mean diff	statistic	p-value	
VMAT1	MDD-	M	7	0.58	0.67	M	0.355	$F_{1,20} = 2.82$	$p = 0.108$
		F	8	0.97	0.44	F	0.388	$F_{1,22} = 1.68$	$p = 0.208$
	MDD+	M	19	0.20	0.33				
		F	20	0.60	0.69				
VMAT2	MDD-	M	7	0.97	0.33	M	-0.158	$F_{1,20} = 0.25$	$p = 0.619$
		F	8	1.33	0.72	F	0.077	$F_{1,22} = 0.07$	$p = 0.788$
	MDD+	M	19	1.08	0.67				
		F	20	1.31	0.65				
PMAT	MDD-	M	7	1.08	0.23	M	-0.045	$F_{1,20} = 0.11$	$p = 0.745$
		F	8	1.24	0.32	F	-0.172	$F_{1,22} = 0.84$	$p = 0.370$
	MDD+	M	19	1.12	0.28				
		F	20	1.40	0.48				
DAT	MDD-	M	7	1.83	1.78	M	-0.195	$F_{1,20} = 0.06$	$p = 0.801$
		F	8	1.37	1.58	F	-0.871	$U = 62^{\dagger}$	$p = 0.359$
	MDD+	M	19	1.17	1.68				
		F	20	2.47	3.05				
NET	MDD-	M	7	0.77	0.57	M	0.183	$F_{1,20} = 0.45$	$p = 0.511$
		F	8	0.57	0.26	F	-0.054	$U = 62^{\dagger}$	$p = 0.360$
	MDD+	M	19	0.78	0.56				
		F	20	0.73	0.45				
SERT	MDD-	M	7	0.65	0.13	M	-0.071	$U = 63^{\dagger}$	$p = 0.840$
		F	8	1.31	1.51	F	0.472	$U = 68^{\dagger}$	$p = 0.542$
	MDD+	M	19	0.66	0.32				
		F	20	0.80	0.42				
TPH1	MDD-	M	7	0.63	0.53	M	-0.295	$F_{1,20} = 2.31$	$p = 0.144$
		F	8	1.06	0.37	F	0.066	$F_{1,22} = 0.14$	$p = 0.707$
	MDD+	M	19	0.97	0.33				
		F	20	0.92	0.34				
TPH2	MDD-	M	7	0.73	0.29	M	-0.095	$F_{1,20} = 0.96$	$p = 0.339$
		F	8	0.85	0.20	F	-0.022	$F_{1,22} = 0.08$	$p = 0.779$
	MDD+	M	19	0.89	0.18				
		F	20	0.95	0.19				

Abbreviations: Antidepressant positive at time of death (+), antidepressant negative at time of death (-), Control (CTRL), standard deviation (sd), Major Depressive Disorder (MDD), number of subjects (N), probability (p). Female (F), Male (M), Non-Suicide (NS), Suicide (S), † Mann-Whitney U Test. Gene names listed in Supplementary Table 1.

(A)

FEMALE

MALE

(B)

FEMALE

MALE

(C)

FEMALE

MALE

Figure 1: Sex differences in the expression of glutamate transporter genes in depression and suicide.

(A) Elevated expression of glutamate transporter genes in depressed females. (B). Lower expression of EAAT1 and higher expression of VGLUT2 in depressed female suicides. (C). Suicide may be an indicator of antidepressant treatment response. Tests of depressed subjects who were antidepressant positive at time of death reveal that female suicides had lower expression of EAAT1 relative to the female non-suicides. In contrast, male suicides who were antidepressant positive, had slightly higher levels of VGLUT2 than male non-suicides.

* $p < 0.05$, ** $p < 0.01$.

(A)

FEMALE

FEMALE

(B)

MALE

MALE

Figure 2: Sex differences in the expression of monoaminergic genes in depression and suicide.

(A) Higher expression of VMAT2, NET and TPH2 in depressed females relative to female controls. (B). Lower expression of VMAT1 and PMAT in depressed males relative to male controls. * $p < 0.05$, ** $p < 0.01$.

(A)

FEMALE

FEMALE

(B)

(C)

MALE

MALE

(D)

(E)

FEMALE

FEMALE

(F)

MALE

MALE

Supplementary Figure 1: No association of monoaminergic gene expression in the DLPFC with suicide or antidepressant status.

(A) and (B) No differences in monoaminergic gene expression between suicides and non-suicides with depression in males or females.

(C) and (D) Suicide in subjects who were antidepressant positive at their time of death may be an indicator of antidepressant treatment response. Tests of depressed subjects who were antidepressant positive reveal no differences in the expression levels of monoaminergic genes between suicides and non-suicides, in males or females.

(E) and (F). The monoaminergic proteins are targets of several conventional antidepressant drugs. Nevertheless, we detect no differences in the expression levels of monoaminergic genes between antidepressant positive and antidepressant negative depressed subjects, in either sex. * $p < 0.05$, ** $p < 0.01$.

Figure 3: Illustration of glutamate transporters within the glutamate synapse.

The three cells within the glutamatergic synapse include the presynaptic neuron, which expresses vesicular transporters (VGLUTs), the postsynaptic neuron, which expresses the majority of glutamate receptors, and the astrocytic end foot. The diagram illustrates the roles and locations of the glutamate transporters without details of postsynaptic density proteins and other molecular pathways. High affinity membrane transporters of glutamate in the glia (EAATs) are responsible for more than 90% of synaptic glutamate reuptake (81) and are essential for reducing synaptic glutamate concentrations. The high concentration of EAATs around synapses ensures that unbound glutamate will encounter available transporters (82). Reduced EAAT protein levels, lead to elevated extracellular glutamate, which leads to increased excitotoxicity and neuronal death (81). One model of MDD suggests decreased synaptic glutamate uptake and a consecutive elevation of extracellular glutamate levels in depression (83). This model and our findings are consistent with reductions in neuronal and glial density observed in post-mortem studies of MDD (84-87). Reduced glutamatergic activity in the DLPFC may explain hypoactivation of this brain region during cognitive and emotional processing tasks in MDD (88-94).