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# Gene expression analysis of novel genes in the prefrontal cortex of major depressive disorder subjects

Dharmendra B. Goswami [a,c,*,1], Courtney S. Jernigan [a,1], Agata Chandran [a], Abiye H. Iyo [a], Warren L. May [b], Mark C. Austin [a], Craig A. Stockmeier [a,d], Beata Karolewicz [a]

[a] Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA
[b] Center of Biostatistics and Bioinformatic, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA
[c] New England Primate Research Center, Harvard Medical School, One Pine Hill Drive, Southborough, MA 01772, USA
[d] Department of Psychiatry, Case Western Reserve University, Cleveland, OH 44106, USA

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ABSTRACT

Dysregulation of the glutamatergic system has been implicated not only in the treatment of major depressive disorder (MDD), but also in the excitotoxic effects of stress and anxiety on the prefrontal cortex, which may precede the onset of a depressive episode. Our previous studies demonstrate marked deficits in prominent postsynaptic proteins involved in glutamate neurotransmission in the prefrontal cortex (PFC), Brodmann's area 10 (BA 10) from subjects diagnosed with major depressive disorder (MDD). In the same group of subjects we have identified deficits in expression and phosphorylation level of key components of the mammalian target of rapamycin (mTOR) signaling pathway, known to regulate translation initiation. Based on our previous findings, we have postulated that glutamate-dependent dysregulation of mTOR-initiated protein synthesis in the PFC may underlie the pathology of MDD. The aim of this study was to use the NanoString nCounter System to perform analysis of genes coding for glutamate transporters, glutamate metabolizing enzymes, neurotrophic factors and other intracellular signaling markers involved in glutamate signaling that were not previously investigated by our group in the PFC BA 10 from subjects with MDD. We have analyzed a total of 200 genes from 16 subjects with MDD and 16 healthy controls. These are part of the same cohort used in our previous studies. Setting our cutoff p-value $\leq 0.01$, marked upregulation of genes coding for mitochondrial glutamate carrier (GC1; $p = 0.0015$), neuropilin 1 (NRP-1; $p = 0.0019$), glutamate receptor ionotropic N-methyl-D-aspartate-associated protein 1 (GRINA; $p = 0.0060$), and fibroblast growth factor receptor 1 (FGFR-1; $p = 0.010$) was identified. No significant differences in expression of the remaining 196 genes were observed between MDD subjects and controls. While upregulation of FGFR-1 has been previously shown in MDD; abnormalities in GC-1, GRINA, and NRP-1 have not been reported. Therefore, this postmortem study identifies GC1, GRINA, and NRP-1 as novel factors associated with MDD; however, future studies will be needed to address the significance of these genes in the pathophysiology of depression and antidepressant activity.



## 1. Introduction

Major depressive disorder (MDD), stress, and anxiety are severe, devastating medical illnesses that affect millions of individuals all over the world. Modern therapeutics have continually relied on the 'monoamine hypothesis' for rational drug design of compounds and still, patients continue to experience low remission rates, residual subsyndromal symptoms, relapses and overall functional impairment. Contrary to this theory, growing evidence indicates that the glutamatergic system has a unique and central role in the neurobiology and treatment of MDD.

*Abbreviations:* MDD, major depressive disorder; PFC, prefrontal cortex; BA 10, Brodmann's area 10; mTOR, mammalian target of rapamycin; GC1, mitochondrial glutamate carrier 1; NRP-1, neuropilin 1; GRINA, glutamate receptor ionotropic N-methyl-D-aspartate-associated protein 1; FGFR-1, fibroblast growth factor receptor 1; NMDA, N-methyl-D-aspartate; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B; NMDAR, N-methyl-D-aspartate receptor; mGluR5, metabotropic glutamate receptor 5; PSD, postsynaptic density; PSD95, postsynaptic density protein 95 kDa; mRNA, messenger RNA; PCR, polymerase chain reaction; Tm, melting temperatures; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; ANCOVA, Analysis of covariance; SHANK2, SH3 and multiple ankyrin repeat domains 2; EAAT-3, Excitatory amino-acid transporters; VGLUT-1, vesicular glutamate transporter; NGFR, nerve growth factor receptor; PKAα, protein kinase A alpha; SLC25A22, solute carrier family 25, member 22; GDH, glutamate dehydrogenase; AnCg, anterior cingulate cortex; DLPFC, dorsolateral PFC; ORB, orbitofrontal cortex; Sema3A, class 3A semaphoring; VEGF, vascular endothelial growth factors; VEGFR, VEGF receptors; PI3K, phosphatidylinositol 3-kinase; Akt, v-akt murine thymoma viral oncogene homolog 1; GSK-3b, glycogen synthase kinase-3beta; BI1, BAX inhibitor-1; TMBIM3, transmembrane BAX inhibitor motif 3; BDNF, brain derived neurotrophic factor; FGF, Fibroblast growth factors.

* Corresponding author at: Department of Psychiatry, University of Mississippi, Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA. Tel.: +1 601 815 5614; fax: +1 601 984 5899.
*E-mail address:* dharmendra_goswami@hms.harvard.edu (D.B. Goswami).
[1] Both authors contributed equally.

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Groundbreaking clinical evidence has been promising, particularly with regard to the N-methyl-D-aspartate (NMDA) antagonist ketamine as a 'proof-of-concept' agent (Mathews et al., 2012). Our group has previously identified robust deficits in prominent postsynaptic proteins involved in glutamate neurotransmission such as N-methyl-D-aspartate receptor (NMDAR) subunits (NR2A and NR2B), metabotropic glutamate receptor 5 (mGluR5), and postsynaptic density protein 95 kDa (PSD95) in the prefrontal cortex (PFC) Brodmann's area 10 (BA 10) from subjects diagnosed with major depressive disorder (MDD) (Deschwanden et al., 2011; Feyissa et al., 2009).

Of particular importance to the cognitive capacities that are uniquely human is the rostral prefrontal cortex, approximating Brodmann's area 10 (BA 10), which is disproportionally larger in humans, relative to the rest of the brain, than it is in the ape's brain (Dreher et al., 2008). BA 10 encompasses the most anterior portion of the frontal cortex, and is most commonly associated with executive functions such as planning and integrative information processing. BA 10 is also connected with the limbic system, making it tempting to speculate that this area is involved in mood regulation. Furthermore, recent mRNA expression and imaging studies indicate altered activity and size of BA 10 in subjects diagnosed with MDD (Altshuler et al., 2008; Monkul et al., 2012; Richieri et al., 2011; Savitz and Drevets, 2009; Shelton et al., 2011).

In our previous group of PFC samples, we have identified deficits in expression and phosphorylation level of key components of the mammalian target of rapamycin (mTOR) signaling pathway, known to regulate translation initiation. Activation of postsynaptic glutamate receptors initiates a cascade which results in mTOR phosphorylation, and eventually, protein synthesis via the downstream effectors of mTOR (Jernigan et al., 2011). Dysregulation of the glutamatergic system may, for this reason, ultimately lead to decreased protein synthesis. Based on our previous findings we have postulated that deficits in synaptic proteins are caused by abnormalities in mTOR signaling, but it is still unclear whether the abnormalities in mTOR signaling precede or follow dysregulation of the glutamatergic system. Recent animal studies have shown that the fast antidepressant response to NMDA receptor antagonists (ketamine and Ro 25–6981) is mediated by rapid activation of the mTOR pathway, leading to an increase in synaptic signaling proteins and increased number and function of new spine synapses in the prefrontal cortex (PFC) of rats (Li et al., 2010). In addition, it has been demonstrated that a single dose of these antagonists rapidly reverses the chronic stress-induced behavioral and synaptic deficits in an mTOR-dependent manner (Li et al., 2010), showing that mTOR-regulated protein synthesis and the glutamatergic system are tightly connected, and that a misbalance of the elemental components of these systems can lead to MDD (Chandran et al., 2012).

From our previous studies, we are confident to claim that the glutamatergic system, through mTOR modulation, plays a pivotal role in MDD. To uncover the key components of this pathway we analyzed genes coding for glutamate transporters, metabolizing enzymes, and various intracellular signal regulators (kinases, neurotrophic factors) which were not previously investigated in our postmortem studies in the PFC (BA 10) using the NanoString nCounter System. By cross-examining these groups of signaling markers, the role of the mTOR pathway components as either the cause or effect glutamatergic dysregulation may be more clearly defined. Cortical tissue samples containing gray matter were dissected from 16 subjects with MDD and 16 healthy controls. These are part of the same cohort used in our previous studies (Deschwanden et al., 2011; Feyissa et al., 2009; Jernigan et al., 2011).

## 2. Methods

### 2.1. Human subjects

Postmortem brain samples were collected at autopsy at the Cuyahoga County Coroner's Office in Cleveland, OH. Informed written consent was obtained from the legal next-of-kin of all subjects. Next-of-kin were interviewed and retrospective psychiatric assessments were conducted in accordance with Institutional Review Board policies at Case Western Reserve University and The University of Mississippi Medical Center. A trained interviewer administered the Schedule for Affective Disorders and Schizophrenia: Lifetime Version (SADS-L) or the Structured Clinical Interview for DSM-IV Psychiatric Disorders (SCID-IV) to knowledgeable next-of-kin to subjects in the study approximately three months after death to determine current and lifetime Axis I psychopathology (Endicott and Spitzer, 1978; First et al., 1996; Spitzer, 1979). Diagnoses for Axis I disorders were assessed independently by a clinical psychologist and a psychiatrist. Consensus diagnosis was reached in conference, using information from knowledgeable informants, The Cuyahoga County Coroner's Office, and all available inpatient and outpatient medical records. Sixteen subjects met criteria for major depressive disorder and sixteen subjects did not meet criteria for an Axis I disorder (termed psychiatrically healthy controls) (Table 1). Among the sixteen depressed subjects, 11 were suicide victims. Blood and urine samples from all subjects were examined by the coroner's office for psychotropic medications and substances of abuse, including ethanol (Tables 1 and 2). There was no evidence of a neurological disorder in any of the subjects. An antidepressant medication (Sertraline) was present in urine of one depressed subject (Tables 1 and 2).

### 2.2. RNA isolation

Total RNA was isolated from PFC BA 10 tissue samples by combination method of Trizol extraction followed by purification using RNeasy columns with DNase 1 treatment (Qiagen, Valencia, CA). PFC tissue was homogenized using a hand held homogenizer with an appropriate volume of Trizol, and the homogenate was kept at RT for 5 min. Chloroform was added to the homogenate and the sample was shaken vigorously for 15 s before allowing the sample tube to sit at room temperature (RT) for 2–3 min. Then the sample tube was spun at $\geq 12{,}000 \times g$ for 15 min at 4 °C. The aqueous phase was carefully removed and transferred to a new tube. The volume of the aqueous sample was measured and an equal volume of 70% ethyl alcohol was added and mixed with a pipette. The sample was loaded into an RNeasy column seated in a collection tube. Precipitate that may have formed was also included. This sample was spun for 30 s at $\geq 8000 \times g$ and flow-through was discarded. This step was repeated until the entire sample had been passed over the column. 350 µl buffer RW1 was added onto the column and spun 30 s at $\geq 8000 \times g$ and at this step we performed the on colume DNAase1 treatment followed by a second 350 µl buffer RW1 wash. The column was transferred into a new collection tube, and 500 µl buffer RPE was added, and the sample was spun for 30 s at $\geq 8000 \times g$ and another 500 µl buffer RPE was added and the sample was spun 2 min at $\geq 8000 \times g$. Flow through was discarded after each step. The column was transferred to a new collection tube and spun (empty) for 1–2 min at $\geq 8000 \times g$. This column was transferred into a new 1.5 ml collection tube and 30–50 µl of RNase-free water was pipetted directly onto the column membrane. The sample was kept at RT for 1–2 min, and then spun 1 min at $\geq 8000 \times g$ to elute the RNA. RNA extracted from PFC was assessed for quantity using Nanodrop 1000 (Nanodrop, Wilmington, DE, US), and for quality using the 2100 Bioanalyzer (Agilent Technologies, Canada). RNA samples were stored in −80 °C until the NanoString assay was performed.

### 2.3. NanoString assay

For our gene expression analysis (200 genes), we utilized the nCounter System from NanoString Technologies, which offers unparalleled performance in digital expression with sensitivity comparable to quantitative polymerase chain reaction (PCR) systems. Unlike microarrays or PCR-based gene expression technologies, the nCounter system does not rely on synthesis of a cDNA strand or PCR amplification,