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

Dharmendra B. Goswami^{a,c,#,*}, Courtney S. Jernigan^{a,*}, Agata Chandran^a, Abiye H. Iyo^a, Warren L. May^b, Mark C. Austin^a, Craig A. Stockmeier^{a,d}, and Beata Karolewicz^a

^aDepartment of Psychiatry and Human Behavior, University of Mississippi Medical Center, 2500 N. State Street, Jackson, MS 39216-4505, USA

^bCenter of Biostatistics and Bioinformatic, University of Mississippi Medical Center, 2500 N. State Street, Jackson, MS 39216-4505, USA

^cNew England Primate Research Center, Harvard Medical School, One Pine Hill Drive, Southborough, MA 01772, USA

^dDepartment of Psychiatry, Case Western Reserve University, Cleveland, OH 44106

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) signalling 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 BA10 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 = 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 (GRIN1; p=0.0060), and fibroblast

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*Correspondence 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. dharmendra_goswami@hms.harvard.edu.

*Both author contributed equally

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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.

Keywords

prefrontal cortex; major depressive disorder; postmortem; gene expression; digital PCR

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. 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, 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) (Feyissa et al., 2009, Deschwanden et al., 2011).

Of particular importance to the cognitive capacities that are uniquely human is the rostral prefrontal cortex, approximating Brodmann's area 10 (BA10), which is disproportionately larger in humans, relative to the rest of the brain, than it is in the ape's brain (Dreher et al., 2008). BA10 encompasses the most anterior portion of the frontal cortex, and is most commonly associated with executive functions such as planning and integrative information processing. BA10 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 BA10 in subjects diagnosed with MDD (Altshuler et al., 2008, Savitz and Drevets, 2009, Richieri et al., 2011, Shelton et al., 2011, Monkul et al., 2012).

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 (Feyissa et al., 2009, Deschwanden et al., 2011, 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, Spitzer, 1979, First et al., 1996). 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 (Table 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 (Table 1 and 2).

2.2 RNA isolation

Total RNA was isolated from PFC BA10 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 seconds before allowing the sample tube to sit at room temperature (RT) for 2–3 min. Then the sample tube was spun at $12,000\times g$ 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 sec at $8,000\times g$ 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 sec at $8,000\times g$ and at this step we performed the on column DNase1 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 sec at $8,000\times g$ and another 500 μl buffer RPE was added and the sample was spun 2 min at $8,000\times g$. Flow through was discarded after each step. The column was transferred to a new collection tube and spun (empty) for 1–2 min at $8,000\times g$. 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 $8,000\times g$ 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, therefore, no enzymes are used in this procedure, removing the bias created by the amplification step. Instead, the barcode-labeled probes anneal directly to mRNAs in solution, and the hybrid molecule is then immobilized, detected and counted. This is a patented technology which gives the true expression of any gene of interest

All custom probe design and synthesis is carried out by Nanostring Technologies as part of the nCounter Gene Expression Assay Kit. Nucleic acids are detected directly in multiplexed probe hybridization reactions using reagents and consumables included in the kit. Target molecules are detected by hybridization to Capture and Reporter Probes, each approximately 50nt in length, and target a contiguous 100-base target region. Target regions of each mRNA

are screened to eliminate direct and inverted repeat elements and evaluated for cross-hybridization against the human RefSeq database. If possible the target region passes across exon-exon boundary. Potential 50-base probes are then selected for melting temperatures (T_m) between 78–83°C. Capture and Reporter Probes are ligated to the synthetic DNA backbones containing the barcode as described (Geiss et al., 2008) and supplied in one tube containing all Capture probes, and one tube containing all Reporter probes. Hybridizations are set up in four pipetting steps as follows: for each tube, sample (100ng of purified RNA or lysate in 5ul total), 10ul hybridization buffer, 10ul supplied Reporter Probe mixture, and lastly 5ul of supplied Capture Probe mixture is added in separate thin-walled PCR strip tubes. The tubes are then covered and incubated at 65°C for 12–18 hours in a thermocycler with heated lid. After hybridization, the samples are processed in the PrepStation and counted in the DigitalAnalyzer. The processing and counting steps are fully automated and require no user interaction. The number of counts of each gene in the CodeSet is output as a comma separated value text file (.csv) that is easily read by Microsoft Excel.

2.4. Data analysis and Statistics

Six positive control nCounter™ spike-ins were used (typically on the order of 10–10,000 counts) to create the calibration curve for each nCounter™ array and eight negative control spike-ins were used to assess the level of background (typically on the order of 1–10 counts). Mean of the negative controls was deducted from all other transcripts in the same assay prior to logarithmic transformation (log base 2); the log values are reported here for simplicity. We used a standard linear regression model to find the least square fit of logarithm-transformed concentration on the logarithm-transformed number of molecules above background to generate the equation for the rest of the transcripts in the same assay. Each nCounter™ assay result was converted to an equivalent concentration using the assay standard curve. Use of the standard curve allows absolute measurements to be assigned to nCounter™ counts as needed. To deduce the precision of the nCounter™ assay itself, we mean centered the data in log2 scale. To achieve specified precision, to generate across-multiple-samples, gene-by-gene equivalency plots, the nCounter™ data were normalized to the geometric mean of reference genes beta2-microglobulin, GAPDH and Tubulin. Comparisons between the groups were made using ANCOVA models that allowed for inclusion of covariates in an effort to adjust for group differences in the non-randomized design. Contrast statements were used to estimate differences between group means on the transformed (base 2 logarithm) scale that, when transformed to the original scale using anti-logs, gave estimates of the ratios of group means. For confounding variance analysis, we used maximum-likelihood mixed-models test to estimate parameters of the models, assuming pairs and subjects within pairs were random components (SAS; Version 9.1, SAS Institute Inc., Cary, NC, USA). As a first step, unadjusted models were fit to compare depressives versus controls without adjusting for potential confounders. Adjusted models included the main effect for comparing depressives versus controls and covariates for age, gender, PMI, and tissue pH. Interactions between the main effect, depressives versus controls, and each of the potentially confounding covariates were investigated and dropped from the model. The covariate adjusted analyses produced similar results; the results for the unadjusted model are reported for simplicity. A p value < 0.01 was considered significant.

3 Results

The nCounter™ technology was used to examine expression levels for 200 genes in tissue dissected from the PFC BA10 from 16 subjects with MDD and 16 matched psychiatrically healthy controls. The results demonstrate marked upregulation of genes coding for mitochondrial glutamate carrier (GC1) in depressed subjects (9.518 ± 0.066) versus healthy controls (9.264 ± 0.042 ; $t=3.398$; $df=30$; $p=0.001$). Likewise, genes coding for neuropilin 1 (NRP-1) were upregulated in depressed subjects (8.192 ± 0.081) versus healthy controls (7.866 ± 0.065 ; $t=3.301$; $df=30$; $p=0.001$). Genes coding for glutamate receptor ionotropic N-methyl-D-aspartate-associated protein 1 (GRINA) were also upregulated in depressed subjects (10.795 ± 0.075) versus healthy controls (10.564 ± 0.036 ; $t=2.892$; $df=30$; $p=0.006$). Fibroblast growth factor receptor 1 (FGFR-1) was upregulated in depressed subjects (8.869 ± 0.087) versus healthy controls (8.595 ± 0.062 ; $t=2.684$; $df=30$; $p=0.010$) as well (Figure 1, Table 3). Based on our rigorous criteria for significance, no differences in expression of the remaining 196 genes were observed between MDD subjects and controls; however we did identify five additional genes which were nearly statistically significant. SHANK2 was slightly reduced in depressed subjects (2.364 ± 0.169) versus healthy controls (2.856 ± 0.142 ; $t=-2.332$; $df=30$; $p=0.024$). EAAT-3 was upregulated in depressive subjects (9.459 ± 0.118) versus healthy controls (9.192 ± 0.088 ; $t=1.894$; $df=30$; $p=0.065$). VGLUT-1 was also increased in depressed subjects (12.821 ± 0.079) versus healthy controls (12.641 ± 0.065 ; $t=1.842$; $df=30$; $p=0.072$). NGFR was reduced in depressive subjects (2.770 ± 0.263) versus healthy controls (3.318 ± 0.135 ; $t=-1.906$; $df=30$; $p=0.064$). PKA- α was slightly increased in depressed subjects (11.424 ± 0.036) versus healthy controls (11.298 ± 0.056 ; $t=1.964$; $df=30$; $p=0.056$). P-values of representative genes from investigated pathways are presented in Table 3.

4 Discussion

In the current study we have identified marked upregulation of genes coding for mitochondrial glutamate carrier-1 (GC-1), neuropilin-1 (NRP-1), glutamate receptor ionotropic, N-methyl D-aspartate-associated protein 1 (GRINA), and fibroblast growth factor receptor 1 (FGFR-1) and moderate upregulation of excitatory amino-acid transporters (EAAT-3), vesicular glutamate transporter (VGLUT-1), nerve growth factor receptor (NGFR) and protein kinase A α (PKA α) in the PFC from subjects diagnosed with MDD as compared to psychiatrically healthy controls. We additionally found a moderate decrease in SH3 and multiple ankyrin repeat domains 2 (SHANK2) in depressed subjects versus healthy controls. While upregulation of FGFR-1 has been previously shown in MDD, no abnormalities in GC1, GRINA, and NRP-1 have been reported to date.

Increased expression of mitochondrial glutamate carrier-1

One of the most highly upregulated genes identified in the current study is SLC25A22 (solute carrier family 25, member 22) which encodes mitochondrial GC-1. The GC-1 protein catalyzes the entry of glutamate into the mitochondrial matrix either with protons (H^+) or in exchange for hydroxyl ions (OH^-) and plays an important role in glutamate metabolism, primarily in astrocytes (Fiermonte et al., 2002, Berkich et al., 2007). Thus, the main physiological role of GC-1 is to import glutamate from the cytosol to the mitochondrial

matrix, where it is oxidized by glutamate dehydrogenase (GDH) to α -ketoglutarate and free ammonia. It has been emphasized that GC is the only mechanism for the supply of external glutamate to the intra-mitochondrial GDH. In our gene expression studies we found a significant upregulation in GC-1 in the PFC with no changes in GDH-1 (Table 3). These results demonstrate, for the first time, that mitochondrial glutamate uptake could be impaired in MDD. In contrast to studies performed in other cortical areas, we have not identified changes in genes coding for cell surface expressed glial transporters (EAAT-1 and EAAT-2) but we notice a modest upregulation of neuronal (EAAT-3) glutamate transporters involved in glutamate uptake from extracellular compartments which did not reach statistical significance (Table 3). Previously, a significant down-regulation of genes coding for EAAT-1 and EAAT-2 was identified in the anterior cingulate cortex (AnCg; BA 24) and left dorsolateral PFC (DLPFC; BA 9 and 46) in subjects with MDD (Choudary et al., 2005). Moreover, deficits in EAAT-1 and EAAT-2 protein immunoreactivity were previously seen in the left orbitofrontal cortex (ORB, BA 47) (Miguel-Hidalgo et al., 2010). Taken, together, these data indicate that dysregulation of key glutamate transporters is brain region-specific and could negatively influence glutamate metabolism/trafficking between neurons and astrocytes (Kelly and Stanley, 2001).

Increased expression of neuropilin-1

For the first time we observed increased NRP-1 expression in depressed brains as compared to controls. NRP-1 is a cell surface receptor that has been implicated in both guidance of neuronal axons and development of the cardiovascular system (Kitsukawa et al., 1997, Kawasaki et al., 1999, Neufeld et al., 2002, Puschel, 2002). The role of NRP-1 in neuronal guidance is mediated by its interaction with a class 3A semaphorin (Sema3A) (Kolodkin et al., 1997). The role of NRP-1 in angiogenesis as well as neurotrophic action is mediated via interaction with vascular endothelial growth factors (VEGF) (Soker et al., 1998, Geretti et al., 2008). NRP-1 enhances VEGF signaling by acting as a coreceptor for VEGF receptors (Geretti et al., 2008), and in concert with VEGF receptors such as flt-1 (VEGFR-1) and flk-1 (VEGFR-2) is frequently described as being involved in the neuroprotective effects of VEGF. We observed increased NRP-1 expression in depressed brains without changes in VEGF (Table 3). To date NRP-1 has not been implicated in the pathology of depression; however NRP-1 signaling partners have been shown to be regulated by stress, antidepressants, and depressive disorder. VEGF has been reported to mediate the neural mechanisms underlying the effects of stress and antidepressants (Warner-Schmidt and Duman, 2007, 2008). It has also been shown that antidepressants increase VEGF expression in the hippocampus (Altar et al., 2004, Warner-Schmidt and Duman, 2007, 2008). Conversely, chronic stress such as cold immobilization, forced cold swimming, or isolation, downregulates the expression of VEGF in hippocampal astrocytes (Heine et al., 2005). These results add to the hypothesis that altered expression of growth factors/neurotrophic factors or associated receptors such as NRP-1 play a significant role in the pathophysiology of depression (Elfving et al., 2010). A possible association between NRP-1 and depressive pathology could be related to aberrant function of downstream signaling effectors (Takahashi et al., 1999, Tamagnone et al., 1999). It has been established that Sema3A signaling through NRP-1 triggers the sequential inhibition of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) leading to activation of glycogen synthase kinase-3 β

(GSK-3b) (Eickholt et al., 2002, Chadborn et al., 2006, Pasterkamp and Giger, 2009). Previously, a significant decrease in Akt and an increase in GSK-3b activities were reported in the ventral PFC (BA 11) in depressed subjects, although no corresponding changes in protein levels were detected (Karege et al., 2007). Similarly, in our study, no group differences were seen in Akt-1 or GSK-3b gene expression levels (Table 3). However, the possibility of dysregulated activities of both Akt and GSK-3b in mood disorders cannot be excluded. Specific inhibitors of GSK-3 have antidepressant behavioral activity (Coyle and Duman, 2003, Gould et al., 2004, Gould et al., 2006). Thus, it is plausible to speculate that increased NRP-1 gene expression in the PFC (BA10) in MDD could be associated with increased GSK-3 activity with no evident alteration in protein or gene expression level. This is consistent with the hypothesis that depression may be associated with impaired inhibitory control of GSK-3. Inhibition of GSK-3 activity could have therapeutic potential for the treatment of mood disorders [reviewed in (Jope, 2011)]. Taken together, aberrant expression and function of NRP-1 could be proposed to trigger neuronal structural changes in neurodevelopmental and psychiatric disorders. Thus, genes associated with NRP-1 signaling such as semaphorins, VEGF, PI3K, Akt, and GSK-3 are likely to represent valuable therapeutic targets to regulate neuronal connectivity, cell death, and synaptic plasticity following nervous system injury and disease.

Increased expression of glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1

In the current study we have identified increased expression of GRIN1 also known as Life guard 1 (Lfg1). GRIN1, initially identified as a 71 kDa glutamate binding subunit (GBP) (Chen et al., 1988, Eaton et al., 1990, Kumar et al., 1991, Ly and Michaelis, 1991) is a member of a protein family characterized by the presence of conserved BAX inhibitor-1 (BI1) motifs (Zhou et al., 2008) embedded in seven transmembrane scaffold (Hu et al., 2009). GRIN1 is localized to neuronal cell bodies (Pal et al., 1999) and dendrites in the brain and spinal cord (Eaton et al., 1990). Because of its structural and functional distinctiveness there were initial doubts as to its identity as a glutamate binding protein (Hollmann and Heinemann, 1994). More recently, it was identified as transmembrane BAX inhibitor motif 3 (TMBIM3) (Nielsen et al., 2011) and is expressed in the brain, at high levels in the hippocampus. Biochemical and sequence analysis of TMBIM3 indicate that rat, murine and human genes encode an approximately 38 kDa protein (Nielsen et al., 2011). Despite numerous studies in the past, the biological function of GRIN1 remains unknown, a TMBIM3 knockout mouse did not have any obvious phenotype (Nielsen et al., 2011). Even though no definite function has been assigned to this gene at the moment, several members of this family of proteins have been shown to protect cells from apoptosis (Xu and Reed, 1998, Fernandez et al., 2007, Gubser et al., 2007). Therefore it is safe to speculate at this point that GRIN1 may also be involved in protecting cells from apoptosis. It is known that depression is associated with structural alterations in limbic brain regions that control emotion and mood (Krishnan and Nestler, 2008, Pittenger and Duman, 2008). Animal models of chronic stress and human postmortem studies have shown that these structural alterations are due to atrophy, loss of neurons, glial cells, decreased neurogenesis and decreased expression of brain derived neurotrophic factor (BDNF) (Duman and Monteggia, 2006, Castren et al., 2007, Pittenger and Duman, 2008, Banasr et al., 2011). In this sense it

is not surprising that our gene expression studies showed a significant increase in the expression of GRINA in the PFC of MDD subjects compared to controls. This increase could possibly occur to counter the obvious neuronal cell loss that is often seen in MDD (Banasr et al., 2011). In addition, disruption of neurotrophic factor expression could contribute to decreased neuroprotection and increased vulnerability to excitotoxicity, resulting in activation of apoptotic pathways (Lucassen et al., 2006). This lends credence to the fact that GRINA, like members of its family of gene products is protective in nature and that the increased expression in this study as alluded to earlier may be to prevent the death of neurons and to encourage neurogenesis. As more studies are carried out on this gene and its product, its true biological function will be elucidated.

Increased expression of fibroblast growth factor receptor 1

Fibroblast growth factors are cell signaling molecules involved in many physiological processes during early development and throughout adulthood, including cell proliferation, migration, differentiation, and survival. Currently, the FGF family in humans comprises 22 ligands and five receptors which are distributed throughout the central nervous system [for review see (Turner et al., 2006, Itoh and Ornitz, 2011)]. We have found a significant increase in FGFR-1 gene expression in the PFC in MDD with no change in FGF-2, FGF-4, FGF-9, FGFR-2, FGFR-3, and FGFR-4 (Table 3). FGFR-1 binds both FGF-1 and FGF-2, is expressed mostly by neurons in the adult, and is widely distributed throughout the central nervous system (Turner et al., 2006). Previously, dysregulation of members of the FGF family have been observed in MDD (Evans et al., 2004, Gaughran et al., 2006, Tochigi et al., 2008). Elevated FGFR-1 gene expression was detected in the PFC from subjects with MDD using DNA microarray analysis (Tochigi et al., 2008). Increased FGFR-1 mRNA expression was also reported in the hippocampus from subjects with MDD using in situ hybridization study (Gaughran et al., 2006). In contrast, one microarray study, found unchanged FGFR-1 expression in the DLPFC and AnCg from subjects with MDD (Evans et al., 2004). Nevertheless, our confirmation of increased levels of FGFR-1 in MDD provides further support to the notion that neurotrophic molecules might have a role in the etiology and treatment of mental disorders.

Reduced expression of SH3 and multiple ankyrin repeat domains 2 (SHANK2)

SHANK2 is a member of the Shank family of synaptic proteins that may function as molecular scaffolds in the postsynaptic density (PSD). Shank proteins contain multiple domains for protein-protein interaction, including ankyrin repeats, an SH3 domain, a PSD-95/Dlg/ZO-1 domain, a sterile alpha motif domain, and a proline-rich region. This particular family member contains a PDZ domain, a consensus sequence for cortactin SH3 domain- binding peptides and a sterile alpha motif. The alternative splicing demonstrated in Shank genes has been suggested as a mechanism for regulating the molecular structure of Shank and the spectrum of Shank-interacting proteins in the PSDs of, both, the mature and developing brain. Two alternative splice variants, encoding distinct isoforms, are reported. Additional splice variants exist but their full-length nature has not been determined (Pruitt et al., 2009, Pruitt et al., 2012). The Shank family of postsynaptic proteins functions as part of the NMDA receptor-associated PSD-95 complex (Naisbitt et al., 1999) Thus, Shank may cross-link Homer and PSD-95 complexes in the PSD and play a role in the signaling

mechanisms of both mGluRs and NMDA receptors (Tu et al., 1999). The nearly significant downregulation of Shank2 mRNA expression in our depressed subjects indicates the alteration in mGluR or NMDAR machinery, which leads to upregulation of downstream pathway molecules, further supporting the importance of the glutamatergic system in depression. Shank 2 has been shown to be highly colocalized with neurofilament, especially dendritic regions, of the neuron. For this reason, a reduction in Shank 2 expression indicates a possible factor in decreased dendritic length, branching, and spines. Shank 2 has not been shown to colocalize to glial fibrillary acidic protein (GFAP)-expressing cells, namely, astrocytes in the developing or adult retina (Kim et al., 2009). For this reason, we speculate that although glia play an integral role in the balance of glutamate and glutamine (Gln) in the central nervous system (Sibson et al., 1998), the role of Shank 2 is neuron-specific.

EAAT-3, VGLUT-1, NGFR and PKA α

In our cohort, we found modest upregulation of EAAT-3, VGLUT-1, and PKA α mRNA; as well as down-regulation of NGFR expression but none reached statistical significance. In neurons, the cellular uptake of glutamate by the excitatory amino-acid transporters (EAATs) decreases excitation and thus confers protection against excitotoxicity. Additionally, mTOR is a powerful regulator of EAAT-3 and may thus contribute a protective effect against neuroexcitotoxicity (Almilaji et al., 2012). The slight increase in EAAT-3 mRNA may indicate an increase in EAAT-3 protein function to compensate for increased glutamate uptake, protecting the neurons in MDD pathology. The vesicular glutamate transporter (VGLUT) is a vesicle-bound, sodium-dependent phosphate transporter that is specifically expressed in the neuron-rich regions of the brain. It is preferentially associated with the membranes of synaptic vesicles and functions in glutamate transport. For this reason, the VGLUT family has a pivotal role in presynaptic release of glutamate into the synaptic cleft (Pruitt et al., 2012). The increase in VGLUT-1 expression indicates an increase in glutamate transporter in MDD, which is opposite to the MDD and Bipolar Disorder study where a decrease in VGLUT1 mRNA expression in entorhinal cortex was identified (Uezato et al., 2009). This difference may be due to different areas of interest between these two studies (prefrontal versus entorhinal cortex). Nerve growth factor receptor (NGFR) was one of the genes from the target list for growth factor receptors and pathways. NGFR is involved in several areas, from axonal guidance signaling and NF- κ B signaling, to induction of apoptosis. The slight decrease in NGFR mRNA expression opposes the finding that during depression the basic cellular machinery moves into hyper drive. Currently there is no other report of NGFR expression changes in human subjects. Protein Kinase A alpha (PKA α), or PRKACA protein kinase, is an important component of intracellular signal transduction cascades that are linked to G-protein coupled receptors and postsynaptic signaling. It is one of the transcription variants of protein kinase A. We found over expression in mRNA which did not reach a statistically significant level. There is only one other human postmortem protein study in BA10 (Shelton et al., 2009) which contrasts with our results but in this contrasting study, total PKA protein levels were measured rather than the individual variant forms.

In the consideration of this data it is, as with all new scientific techniques, important to proceed with caution. As research techniques evolve it is possible to achieve more precise

results in a variety of applications, and consideration of cell populations in such precise results is of great value. In this study we used whole tissue punches rather than laser capture microdissection, also called microdissection, laser microdissection, or laser-assisted microdissection, which could have allowed us to gain access to a more purified single cell type population. Even though our samples consist of a heterogeneous population of cells from BA 10 region, the expression changes we reported are very strong. This indicates that the changes observed in this study are, in fact, real changes across the BA 10 region in MDD. In the future it will be of great value to follow this study with the analysis MDD-induced changes in individual cell types in order to further investigate the function of the statistically significant mRNA expression levels as well as the near- and non-significant mRNA levels, as they relate to specific cell populations.

In conclusion, our results indicate that glutamate transport in the mitochondria may be impaired in MDD. In addition to this novel finding, neurotrophic factors and signal mediators might also be altered in MDD pathology. In this study we have shown a clear perturbation in signaling molecules associated with the glutamatergic system which are important for normal functioning of the brain. The present findings identify GC1, GRINA, NRP-1, and FGFR-1 as potential key players in either the etiology or the expression of severe depression and suggest new strategies for developing treatments for this disease. Since many of the genes of interest from this study have already been characterized in vitro, these studies may be followed with established glutamate-dependent cellular modifications; however, animal models will be an integral part of future research to test the role of these genes in depressive-like behavior and antidepressive activity.

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Abbreviation

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-3β	glycogen synthase kinase-3beta
BI1	BAX inhibitor-1
TMBIM3	transmembrane BAX inhibitor motif 3
BDNF	brain derived neurotrophic factor
FGF	Fibroblast growth factors

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Figure 1.

Expression of GC1, NRP-1, FGFR-1 and GRINA in the PFC BA10 from subjects with MDD and psychiatrically normal controls. The nCounter™ data were normalized to geometric mean of reference gene beta2-microglobulin, GAPDH and Tubulin gene expression as described in the Methods section. Normalized values for the individual subjects and mean values (horizontal lines) are presented. Significant increases in GC1, NRP-1, FGFR-1 and GRINA were observed in depressed subjects (filled circles; $n=16$) as compared to controls (open circle; $n=16$).

Table 1

Case demographics of controls and major depressive subjects.

Control					MDD					
#	Age ^	Sex	PMI	pH	Toxicology	Age ^	Sex	PMI	pH	Toxicology
1	35	M	25	6.74	Lidocaine	42	M	44.5	6.67	Clean
2	30	M	19	6.98	Clean	41	M	19.25	6.24	Chlorpheniramine
3	33	M	23	6.86	Clean	30	M	18	6.91	Clean
4	37	F	13	5.93	Clean	34	F	24	6.27	Ethanol, CO
5	51	F	22	6.3	Clean	40	F	25	6.32	Morphine, codeine
6	54	M	17	6.87	Brompheniramine	50	F	28.5	6.47	Dextromethorphan
7	59	M	6	6.79	Lidocaine	60	M	20	6.31	Ethanol
8	34	M	24	6.61	Ethanol	42	M	20	6.8	Clean
9	54	M	19	6.52	Lidocaine	52	M	17	6.48	CO
10	52	M	17	6.28	Clean	48	M	21	6.9	Flurazepam
11	48	M	9	6.98	Clean	65	M	30	6.24	Codeine
12	74	M	21	6.62	Clean	81	M	33	6.78	Clean
13	65	F	26	6.17	Clean	43	F	24	6.40	Lidocaine
14	80	F	21	6.78	Clean	82	M	12	6.46	CO
15	54	M	26.5	6.5	Morphine	42	M	20	6.64	Sertraline
16	61	M	30.5	6.61	Lidocaine	42	F	7.25	5.62	Clean
Mean	51.3		19.9	6.5		49.6		22.7	6.4	
SD	3.6		1.6	0.07		3.7		2.1	0.08	

M, male; F, female; PMI, postmortem interval (hours);

[^] Age in years; MDD, major depressive disorder; CO, Carbon Monoxide Poisoning

Table 2

Summary of demographic characteristics of subjects

Parameter	Controls (n=16)	Major Depression (n=16)
Age *	51.31±3.69	49.63±3.80
Postmortem Interval *	19.94±1.62	22.72±2.16
pH *	6.60±0.08	6.47±0.08
Gender (female/male)	4/12	5/11
Medication History [†]	None	Sertraline (n=3) Fluoxetine (n=1) Paroxetine (n=1) Trazedone (n=1) Imipramine (n=1) Risperidone (n=1)
Comorbid Diagnosis	Ethyl Alcohol Intoxication (n=1) History of Ethyl Alcohol Dependence (n=1)	Ethyl Alcohol Intoxication (n=5) History of Ethyl Alcohol Dependence (n=3) Anxiolytic Intoxication (n=4) Opiate Intoxication (n=1) Cannabis Dependence (n=1)
Smoking	Dependent (n=6) History of Dependence (n=2) Occasional (n=1) None (n=6)	Dependent (n=8) None (n=8)
Suicide	None	n=11

* Mean±SEM

[†] Prescriptions for antidepressants within 4 weeks prior to death; one of the 16 depressed subjects had antidepressants (sertraline) present in their postmortem toxicology screening.

Table 3

Representative genes from investigated pathways

Category	Genes	Transcript accession numbers	Description	p value
Glutamate transporters	EAAT-1	NM_004172	SLC1A3 solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.928
	EAAT-2	NM_004171	SLC1A2 solute carrier family 1 (glial high affinity glutamate transporter), member 2	0.418
	EAAT-3	NM_004170	SLC1A1 solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, member 1	0.065
	VGLUT-1	NM_020309	SLC17A7 solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	0.072
	GC-1	NM_001191060	SLC25A22 solute carrier family 25 (mitochondrial carrier: glutamate), member 22	0.001*
Glutamate metabolizing enzymes	GS	NM_001033056	GLUL glutamate-ammonia ligase	0.464
	GDH-1	NM_005271	GLUD1 glutamate dehydrogenase 1	0.987
	GLS	NM_014905	Glutaminase	0.130
Neurotrophic factors and associated receptors	BDNF	NM_170731	Brain-derived neurotrophic factor	0.871
	TrkA	NM_002529	NTRK1 neurotrophic tyrosine kinase, receptor, type 1	0.424
	TrkB	NM_006180	NTRK2 neurotrophic tyrosine kinase, receptor, type 2	0.188
	FGF-2	NM_002006	Fibroblast growth factor 2 (basic)	0.648
	FGF-4	NM_002007	Fibroblast growth factor 4	0.284
	FGF-9	NM_002010	Fibroblast growth factor 9	0.393
	FGFR-1	NM_023110	Fibroblast growth factor receptor 1	0.010*
	FGFR-2	NM_000141	Fibroblast growth factor receptor 2	0.802
	FGFR-3	NM_000142	Fibroblast growth factor receptor 3	0.578
	FGFR-4	NM_002011	Fibroblast growth factor receptor 4	0.878
	VEGF	NM_001025366	VEGFA vascular endothelial growth factor A	0.201
	NRP-1	NM_001024629	Neuropilin 1	0.001*
	NGF	NM_002506	Nerve growth factor	0.149
	NGFR	NM_002507	Nerve growth factor receptor	0.064
Other signaling markers	Shank-2	NM_012309	SH3 and multiple ankyrin repeat domains 2	0.024
	SAP-97	NM_001098424	DLG1 discs, large homolog 1	0.191
	Homer1b	AF093262	Homo sapiens homer homolog 1b	0.259
	PSD-93	NM_001142699	DLG2 discs, large homolog 2	0.130
	GRINA	NM_000837	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1	0.006*
	PKA α	NM_002730	PRKACA protein kinase, cAMP-dependent, catalytic, alpha	0.056
	PKC α	NM_002737	PRKCA protein kinase C, alpha	0.066

Category	Genes	Transcript accession numbers	Description	p value
	Akt-1	NM_005163	v-akt murine thymoma viral oncogene homolog 1	0.231
	GSK-3B	NM_002093	Glycogen synthase kinase 3 beta	0.141
	ERK-1	NM_002746	MAPK3 mitogen-activated protein kinase 3	0.221