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Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression

RC Shelton[1], J Claiborne[1], M Sidoryk-Wegrzynowicz[2], R Reddy[1], M Aschner[2], DA Lewis[3] and K Mirnics[4]

[1]*Department of Psychiatry, Vanderbilt University School of Medicine, Nashville, TN, USA;* [2]*Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN, USA;* [3]*Departments of Psychiatry and Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA and* [4]*Department of Psychiatry and the John F. Kennedy Center for Human Development, Vanderbilt University, Nashville, TN, USA*

The etiology of major depression (MDD), a common and complex disorder, remains obscure. Gene expression profiling was conducted on post-mortem brain tissue samples from Brodmann Area 10 (BA10) in the prefrontal cortex from psychotropic drug-free persons with a history of MDD and age, gender, and post-mortem interval-matched normal controls ($n = 14$ pairs of subjects). Microarray analysis was conducted using the Affymetrix Exon 1.0 ST arrays. A set of differential expression changes was determined by dual-fold change-probability criteria (laverage log ratios| > 0.585 [equivalent to a 1.5-fold difference in either direction], $P < 0.01$), whereas molecular pathways of interest were evaluated using Gene Set Enrichment Analysis software. The results strongly implicate increased apoptotic stress in the samples from the MDD group. Three anti-apoptotic factors, Y-box-binding protein 1, caspase-1 dominant-negative inhibitor pseudo-ICE, and the putative apoptosis inhibitor FKGS2, were over-expressed. Gene set analysis suggested up-regulation of a variety of pro- and anti-inflammatory cytokines, including interleukin 1α (IL-1α), IL-2, IL-3, IL-5, IL-8, IL-9, IL-10, IL-12A, IL-13, IL-15, IL-18, interferon gamma (IFNγ), and lymphotoxin α (TNF superfamily member 1). The genes showing reduced expression included metallothionein 1M (MT1M), a zinc-binding protein with a significant function in the modulation of oxidative stress. The results of this study indicate that post-mortem brain tissue samples from BA10, a region that is involved in reward-related behavior, show evidence of local inflammatory, apoptotic, and oxidative stress in MDD.
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Keywords: major depression; microarray; inflammation; apoptosis; cytokines; oxidative stress

Introduction

A variety of brain imaging, post-mortem morphometric, and other studies suggest that major depression (MDD) involves abnormalities of cortico-limbic structures, including prefrontal cortex, anterior cingulated, amygdala, and hippocampus.[1] In spite of the fact that there is considerable evidence suggesting an underlying neurobiology for MDD, the molecular basis has been elusive. A number of gene expression profiling studies in post-mortem brain tissue have been conducted comparing depressed and control samples, with variable results.[2–9] Transcriptome profiling is a powerful, albeit complex, method, which is affected by a number of factors, including accuracy of the pre-mortem diagnosis; peri-mortem characteristics such as cause of death, the presence of prolonged agonal states, the presence of drugs or toxins;[10,11] post-mortem factors such as the post-mortem interval (PMI), pH, and RNA integrity; processing methods; and other issues. Even when controlling for all of these variables, contemporary microarrays provide enormous amounts of data, creating challenges in data handling. Further, MDD is not a unitary condition and is likely to comprise a range of clinical phenotypes and endophenotypes, which may affect the results of microarray analysis.

This study was aimed at conducting gene expression analysis of a brain area, Brodmann Area 10 (BA10), which was chosen for several reasons. First, BA10 has been shown to be significantly involved in the mediation of reward-related behavior, which is central to the clinical phenomenology of depression.[12] For example, Rogers *et al.*[12] showed that a task, which involved choices between high probability and low reward versus low probability and high reward options resulted in the activation of three adjacent regions in the orbito-frontal cortex, BA10, BA11, and BA47 using [^{15}O] positron emission tomography. Cocaine[13] and amphetamine,[14] highly

Correspondence: Dr RC Shelton, Department of Psychiatry, Vanderbilt University School of Medicine, 1500 21st Avenue, South, Suite 2200, Nashville, TN 37212, USA.
E-mail: richard.shelton@vanderbilt.edu
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reinforcing substances, have also been shown to produce activation of BA10. Kufahl *et al*.[13] showed that cocaine administration resulted in decreased fMRI brain oxygen-level-dependent signal in ventral tegmental area, nucleus accumbens, subcallosal cortex, ventral pallidum, amygdala, parahippocampal gyrus, posterior orbital gyrus, inferior and superior temporal gyrus, but marked and sustained increases in brain oxygen-level-dependent signal in BA10 and 11. Similarly, amphetamine administration increased BA8 and 10 activities as measured by single photon emission tomography.[14] These data suggest that BA10 is involved in reward and reinforcement processing, a core dysfunction in depression.[15,16]

Only one earlier study has evaluated gene expression in post-mortem tissue samples from BA10 in MDD compared with controls.[17] This study found 99 differentially expressed genes, which included sets of transcripts of genes involved in cell proliferation. Most of the altered genes are of unknown significance with regard to MDD. However, one up-regulated transcript, fibroblast growth factor receptor 1 (*FGFR1*), may be significant; the expression of both *FGFR1*[18] and its endogenous ligands *FGF1* and *FGF2*[3] had earlier been shown to be altered in MDD samples.

This study investigated post-mortem brain tissue samples from 14 depressed persons who were psychotropic drug free at the time of death and age- and sex-matched normal controls. Eleven of the 14 samples were from persons who met full DSM-IV criteria for MDD, melancholic subtype antemortem. The melancholic subtype is a distinct clinical phenotype, characterized by symptoms of hyperarousal (for example insomnia and anorexia) and hypothalamic–pituitary–adrenal axis activation[19] that may persist even after successful treatment.[20] The Affymetrix Exon 1.0 ST array was used for assessing expression of gene transcripts using mRNA of high integrity. In addition to the gene expression analysis, molecular pathways of interest were evaluated using Gene Set Enrichment Analysis (GSEA) to determine altered expression of sets of genes implicated in depression. The results showed altered expression of a large number of genes, but specifically showed up-regulation of the expression of pro- and anti-inflammatory cytokines and specific anti-apoptotic factors.

Materials and methods

Human brain samples and tissue preparation

All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research and the Vanderbilt University Health Sciences Institutional Review Board. Fresh-frozen brain specimens were obtained during routine autopsies conducted by a medical examiner after consent for tissue donation was given by the next of kin. Samples were obtained from 14 pairs of persons with major depressive disorder (MDD) and control (CTR) post-mortem brains matched for sex and race, and as closely as possible for age (MDD = 47.2 [s.d. = 14.0] and CTR = 48.9 [s.d. = 12.1], respectively), and PMI (17.8 [s.d. = 7.6] hours and 17.8 [s.d. = 6.5] hours, respectively) (all values mean ± s.d.) (Table 1). Depressed and control subject brain pH (6.59 [s.d. = 0.30] and 6.71 [s.d. = 0.26], respectively) and RNA integrity number (> 7 for all subjects) were also assessed (Table 1). There were no statistically significant differences in age, PMI, pH, or RNA integrity number between groups. All samples came from persons free from all known psychotropic agents and recent substance abuse. Blood samples for toxicology were obtained at the time of the brain collection; all participants were confirmed to be free of psychotropic medications except for one control subject who tested positive for temazepam. Toxicology results are listed in Table 1. The brain samples were collected through the local medical examiner's office and all patients died a non-hospitalized, acute death, with virtually no agonal state that could bias the gene expression measurements.

Field evaluations were conducted by trained and experienced clinicians and consisted of structured interviews with family members of the deceased. Written informed consent was obtained from all participants. After field interviews were completed, a committee of experienced research clinicians performed an independent diagnostic conference and assigned consensus DSM-IV diagnoses (including depressive subtypes) for each subject on the basis of medical records and the results of structured interviews conducted with family members of the deceased. Eleven of the 14 depressed samples came from persons with the melancholic subtype.

On receipt, the brains were dissected coronally, flash frozen, and stored at −80 °C. An ∼5 mm × 5 mm gray matter cube from BA10 (spanning the cortical gray matter) was carved out from the frozen block using pre-chilled dental tools. This ensured a more homogenous sample, greatly diminishing harvesting bias (variable white matter/gray matter ratio) and reducing experimental noise in our dataset. Furthermore, this approach allowed us to preferentially detect expression changes in neuron- and glia-enriched tissue. The tissue piece was immediately placed in cold Trizol reagent, homogenized and used for RNA extraction.[21]

Design and processing of RNA microarrays

RNA concentration was determined with an Agilent BioAnalyzer 2100 system (Agilent, Palo Alto, California, USA).[21] The RNA integrity number was > 7 for all subjects. cDNA synthesis, amplification, and labeling were performed using the manufacturer's protocol (http://www.affymetrix.com/support/technical/technotes/human_exon_wt_target_technote.pdf). Briefly, the labeling started with a ribosomal RNA reduction step, in which 60–80% of the ribosomal RNAs are eliminated. cDNA is generated by a random priming method. The random primers incorporate a T7 promoter sequence, which is subsequently used in

Table 1 Sample characteristics

Subject number	*Group*	*Pair*	*Sex*	*Race*	*Age*	*PMI (h)*	*pH*	*Cause of death*	*Manner of death*	*Blood toxicology*
600	MDD	1	M	W	63	9.9	6.72	Hanging	Suicide	None
613	MDD	2	M	W	59	15.6	6.95	Gunshot wound	Suicide	None
668	MDD	3	M	W	34	24.3	7.00	Hanging	Suicide	None
699	MDD	4	M	W	65	5.5	6.71	Gunshot wound	Suicide	None
735	MDD	5	F	W	40	14.0	6.84	Pulmonary embolism	Accidental	None
927	MDD	6	M	W	58	24.9	6.11	ASCVD	Natural	None
949	MDD	7	M	W	38	25.0	6.23	Cardiac arrhythmia	Natural	None
1028	MDD	8	M	W	39	14.5	6.18	Gunshot wound	Suicide	None
1053	MDD	9	M	W	47	24.0	6.57	ASCVD	Natural	Lidocaine
1131	MDD	10	M	W	29	26.6	6.92	Gunshot wound	Suicide	None
1186	MDD	11	M	W	45	6.6	6.25	Traumatic asphyxiation	Accidental	None
1215	MDD	12	M	W	44	11.0	6.54	ASCVD	Natural	None
1221	MDD	13	F	B	28	24.8	6.61	Pulmonary embolism	Natural	None
10028	MDD	14	F	W	72	23.1	6.66	Gunshot wound	Suicide	Ibuprofen
MDD mean (s.d.)					47.2 (14.0)	17.8 (7.6)	6.59 (6.46)			
510	CONTROL	1	M	W	63	12.4	6.51	GI hemorrhage	Natural	None
685	CONTROL	2	M	W	56	14.5	7.06	Coronary artery disease	Natural	Alcohol (0.01%)
694	CONTROL	3	M	W	38	20.7	6.73	Subarachnoid hemorrhage	Natural	None
615	CONTROL	4	M	W	62	7.2	6.39	Ruptured aortic aneurysm	Natural	None
567	CONTROL	5	F	W	46	15.0	6.77	Mitral valve prolapse	Natural	None
902	CONTROL	6	M	W	60	23.6	6.74	ASCVD	Natural	None
700	CONTROL	7	M	W	42	26.1	6.95	ASCVD	Natural	None
1047	CONTROL	8	M	W	43	13.8	6.63	ASCVD	Natural	Chlorphen-iramine
643	CONTROL	9	M	W	50	24.0	6.23	ASCVD	Natural	None
789	CONTROL	10	M	W	22	20.0	7.04	Asphyxiation	Accidental	None
1067	CONTROL	11	M	W	49	6.5	6.55	Hypertensive heart disease	Natural	Doxylamine, metoprolol, temazepam
857	CONTROL	12	M	W	48	16.6	6.54	ASCVD	Natural	None
1282	CONTROL	13	F	W	39	24.5	6.84	Cardiac arrhythmia	Natural	None
818	CONTROL	14	F	W	67	24.0	7.06	Anaphylaxis	Accidental	None
Control mean (s.d.)					48.9 (12.1)	17.8 (6.5)	6.71 (0.25)			

Abbreviations: MDD, major depression; PMI, post-mortem interval.

an *in vitro* transcription to produce anti-sense cRNA fragments, in which a modified dUTP is incorporated instead of dTTP. The modified dUTP is subsequently recognized by the enzymes Urasil-DNA glycosylase and human apurinic/apyrimidinic endonuclease 1, which will cut the DNA, resulting in fragmentation of the cDNA. Each DNA fragment is end labeled with biotin using terminal deoxynucleotidyl transferase before being hybridized to the arrays.

In this study, we used the Human Affymetrix Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA); for this study, we will report the analyses for the 60 000 core transcripts. Hybridization to the Human Exon 1.0 ST array, post-hybridization washes, staining, array image generation, segmentation, and QC analysis were performed by the vanderbilt microarray shared resource using the manufacturer's standard protocols.

Identifying differentially expressed genes

After a visual inspection of unsegmented microarray scans, standard image segmentation was performed and DAT files were generated. CEL files produced by the vanderbilt microarray shared resource were imported into Expression Console software, version 1.1 (Affymetrix), and data normalized using the robust multi-array averaging algorithm. Export of the data from the Expression Console using the 'Core' data filter collapsed exon-level data onto known transcripts, producing $\sim$60 000 transcript-level data points for each subject. Data were analyzed in Excel (Microsoft, Redmond, WA, USA) in two steps. A Student's groupwise two-tailed *t*-test compared robust multi-array averaging intensities of MDD and controls samples. Then, signal intensity magnitude difference was calculated as a $\log_2$-based average log ratios (ALR). ALR is calculated according to the following equation:

$$\mathrm{ALR} = \mathrm{mean}(\log_2\{\mathrm{D}_1\}\ldots\log_2\{\mathrm{D}_n\}) - \mathrm{mean}(\log_2\{\mathrm{C}_1\}\ldots\log_2\{\mathrm{C}_n\}),$$

where D = depressed and C = control individual gene expression values. As gene expression differences of miniscule magnitude often may have statistical significance (but represent mostly artifacts), we used a conservative, earlier established dual-criteria approach[21–24] to identify the differentially expressed genes in our dataset: a gene was considered differentially expressed if it showed an $|\mathrm{ALR}| > 0.585$ (representing a 1.5-fold [50%] difference in either direction) and a groupwise $P < 0.01$ between MDD and control subjects.

Clustering

Hierarchical clustering was performed to identify similarities in gene expression change (potentially co-regulated genes) and to assess the capacity of the disease gene 'signature' to dichotomize the subject population into disease and control groups (that is does the disease gene signature adequately 'diagnose' the disease). Differentially expressed qgenes ($\mathrm{ALR} > 0.585$, $P < 0.01$) were subjected to an unsupervised two-way clustering analysis based on Euclidian distance (robust multi-array averaging-normalized expression profile for individual genes $\times$ individually analyzed samples) using GenePattern[25] as described earlier.[21]

Correlation analysis

Bivariate correlations were determined using the Pearson Product-Moment correlation coefficient (SPSS version 16, SPSS, Chicago, IL, USA). robust multi-array averaging-normalized ALR values were used as input data.

Gene set analysis

Gene set analysis was performed using GSEA software as described earlier.[26] This method determines whether *a priori* defined set of genes shows statistically significant, concordant differences between two biological states or phenotypes. The molecular signatures database contains >3000 knowledge-based gene sets for use with GSEA, and the dataset is analyzed simultaneously for multiple gene sets.[27] To simultaneously analyze for gene set enrichment and control for false discovery, we performed 1000 permutations per analysis using the MDD versus control phenotypes.[27] False discovery rate threshold in GSEA was measured by *Q*-value, a test that measures the proportion of false positives incurred when that particular test is called significant. *Q*-value was set at $q \leqslant 0.05$,[28] identifying differentially expressed gene sets that had >95% probability to represent true, biological observations. Enrichment sets represent BioCarta-derived (http://www.biocarta.com/genes) gene sets (designated as 'pathways'), corrected for multiple comparisons. The enrichment score represents the degree to which a specific set is represented at the extremes (high or low) of the entire list. The enrichment score corresponds to a weighted Kolmogorov–Smirnov-like statistic.[27] The statistical significance (nominal *P*-value) was calculated using permutation testing. The core members of high scoring gene sets that contribute to the enrichment score are extracted by defining the subset of genes in the set that appear in the ranked list at or before the point in which the running sum reaches its maximum deviation from zero. This is the subset of a gene set that accounts for the enrichment signal.[27]

Real-time quantitative polymerase chain reaction

Ten genes, showing differential expression between MDD and CTR subjects, were selected for quantitative polymerase chain reaction (qPCR) analysis. For each of the selected genes, cDNA was synthesized with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, California, USA). TaqMan gene expression assays (Applied Biosystems) were performed on 50 ng cDNA/sample for 10 genes: histone cluster 1, H2aj (HIST1H2AJ), Y-box-binding protein 1 (YBX1), caspase-1 dominant-negative inhibitor pseudo-ICE (COP1), Zinc Finger Protein 117 (ZNF117), Casein α S1 (CSN1S1), Calponin 3, acidic

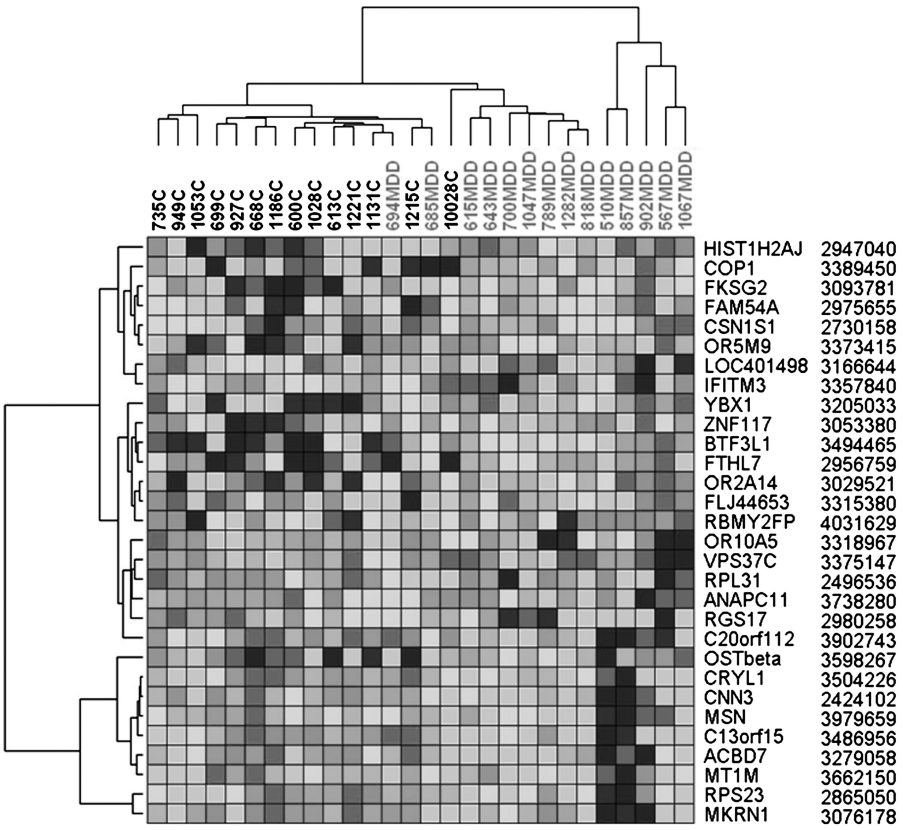

Figure 1 Hierarchical clustering of differentially expressed genes. Normalized log2 intensities were clustered by GenePattern in two dimensions (horizontal: genes; vertical: samples [gray = MDD, black = controls]) on the basis of Euclidian distance. Each colored pixel represents a single gene expression value in one subject. The color intensity is proportional to its relative expression level (blue: under-expressed; red: over-expressed). Note that the statistical segregation of depressed and control subjects are almost complete, with an overlap of only two controls. Labels on the right denote gene symbols and probe identifiers. For more data, see Table 2. (The color reproduction of this figure is available on the html full text version of the manuscript.)

(CNN3), olfactory receptor, family 10, subfamily A, member 5 (OR10A5), vacuolar protein sorting 37 homolog C (*Saccharomyces cerevisiae*) (VPS37C), Metallothionein 1M (MT1M), and Moesin (MSN). The results were then verified and normalized against endogenously glyceraldehyde-3-phosphate dehydrogenase, which did not display a significant gene variation between MDD and CTR subjects. The qPCR reactions were performed by an ABI Prism 7000 SDS cycler (Applied Biosystems) with the ABI Prism 7000 SDS software. The software was run with the automatic baseline and threshold (Ct) detection options selected. The quantified qPCR data was exported to Microsoft Excel to organize MDD and CTR ΔCt, and determine $MDD_{\Delta Ct} - CTR_{\Delta Ct}$ (ΔΔCt).

Data sharing
Complete microarray data will be made available by request on publication.

Results

The Affymetrix Human Exon 1.0 array allowed us to analyze 60 000 gene expression products. Samples from MDD and CTR subjects were obtained from BA10 in the prefrontal cortex. Our analysis revealed 30 transcripts, which displayed significant expression differences between MDD and CTR samples, using a metric of $|ALR| > 0.585$ (50% change) and $P \leqslant 0.01$ (Figure 1). Of these genes, 14 genes (46.7%) were found to be higher in MDD samples compared with CTR samples with a mean ALR of 0.80 (1.7-fold increase; Table 2) and 16 genes (53.3%) were found to be under-expressed in MDD as compared with CTR samples with a mean ALR value of −0.70 (1.6-fold decrease; Table 2).

qPCR findings
Ten genes were selected to validate our microarray findings. These transcripts were selected for validation because of their putative function in dysregulation of cytokine pathways in MDD as compared with CTR subjects. Five of the gene transcripts were over-expressed (HIST1H2AJ, YBX1, COP1, ZNF117, CSN1S1) and the other five were under-expressed (CNN3, OR10A5, VPS37C, MT1M, MSN) in the MDD samples. In addition, one over-expressed cytokine, interleukin 9 (IL-9) was also included. For these 11

Table 2 Genes with increased and decreased expression in BA10 of subjects with major depressive disorder

UniGene	*Gene name*	*Gene symbol*	*Chromosome*	*ALR*[a]	P[b]
(A) Increased expression					
Hs.406691	Histone cluster 1, H2aj	HIST1H2AJ	6p22-21.3	1.577	<0.0001
Hs.473583	Y-box-binding protein 1	YBX1	1p34	1.136	0.0014
Hs.348365	Caspase-1 dominant-negative inhibitor pseudo-ICE	COP1	11	0.996	0.0036
Hs.534533	Organic solute transporter beta	OSTbeta	15q22.31	0.819	0.0015
Hs.3155	Casein α s1	CSN1S1	4q21.1	0.778	0.0070
Hs.651853	Apoptosis inhibitor	FKSG2	8p11.2	0.741	0.0066
Hs.567241	Basic transcription factor 3, like 1	BTF3L1	13q22	0.688	0.0066
Hs.250693	Zinc finger protein 117	ZNF117	7q11.21	0.684	0.0005
Hs.534547	Olfactory receptor, family 2, subfamily A, member 14	OR2A14	7q35	0.671	<0.0001
Hs.121536	Family with sequence similarity 54, member A	FAM54A	1q32.3	0.664	0.0065
Hs.660426	FLJ44653 protein	FLJ44653	10q26.3	0.653	0.0002
Hs.684794	RNA-binding motif protein, Y-linked, family 2, member F pseudogene	RBMY2FP	Yq11.223	0.624	0.0085
Hs.333125	Ferritin, heavy polypeptide-like 7	FTHL17	Xp21	0.622	0.0004
Hs.553749	Olfactory receptor, family 5, subfamily M, member 9	OR5M9	11q11	0.592	0.0007
(B) Decreased expression					
Hs.166313	Regulator of g-protein signalling 17	RGS17	6q25.3	−0.924	0.0012
Hs.534456	APC11 anaphase promoting complex subunit 11	ANAPC11	17q25.3	−0.912	0.0064
Hs.516978	Chromosome 20 EST	C20orf112	20q11.1-11.23	−0.901	0.0095
Hs.647370	Metallothionein 1M	MT1 M	16q13	−0.864	0.0104
Hs.644598	Acyl-coenzyme a binding domain containing 7	ACBD7	10p13	−0.804	0.0056
Hs.646477	interferon-induced transmembrane protein 3	IFITM3	7p11.2	−0.779	0.0049
Hs.490347	Makorin, ring zinc finger protein, 1	MKRN1	7q34	−0.690	0.0092
Hs.507866	Chromosome 13 open reading frame 15	C13orf15	13q14.11	−0.672	0.0097
Hs.87752	Moesin	MSN	Xq11.2-q12	−0.686	0.0025
Hs.469473	Ribosomal protein L31	RPL31	2q11.2	−0.630	0.0087
Hs.523715	Vacuolar protein sorting 37 homolog C	VPS37C	11q12.2	−0.620	0.0084
Hs.447478	Olfactory receptor, family 10, subfamily A, member 5	OR10A5	11p15.4	−0.606	0.0059
Hs.527193	Ribosomal protein s23	RPS23	5q14.2	−0.605	0.0091
Hs.483454	Calponin 3, acidic	CNN3	1p22-p21	−0.603	0.0039
Hs.370703	Crystallin, λ 1	CRYL1	13q12.11	−0.589	0.0043
Hs.522063	RIKEN A930001M12	LOC401498	9p21.1	−0.588	0.0017

Abbreviations: ALR, average log ratios; BA10, Brodmann Area 10.
[a]Mean ALR, depressed minus control.
[b]Groupwise *P*-value.

transcripts, the ΔΔCt qPCR findings were highly correlated ($r=0.92$; $P<0.001$) with the microarray data between MDD and CTR subjects (Figure 2).

Effects of age, sex, pH, and post-PMI on expression levels

Correlations between pH, PMI, age, and differentially expressed genes were performed. A significant correlation was found only between pH and the expression level of ZFN117 ($r=0.411$, d.f. = 28, $P=0.03$); after correction for multiple comparisons, this was no longer significant. All differentially expressed genes were compared by sex using the Student's *t*-test; all comparisons were not significant.

Correlational analysis

Associations between the selected genes differentially expressed in MDD were tested using the Pearson Product Moment correlation coefficient. Genes for this analysis were selected based on their effects on transcriptional regulation (HIST1H2AJ, ZNF117, MKRN1, RGS17), apoptosis (YBX1, COP1, FKSG2), structural integrity (MSN), or modulation of oxidative stress (MT1M). Most genes showed significant direct or inverse correlations with other differentially expressed genes. There were particularly strong correlations ($r>0.600$) found between MT1M, MKRN1, and MSN, all with reduced expression. In addition, the anti-apoptotic factors YBX1, COP1, and FKSG2 were significantly correlated with each other, and with the histone protein HIST1H2AJ.

Gene set enrichment analysis

Of the eight pathways, which showed significant enrichment (Table 3), the most robust finding revealed an up-regulation of transcripts pertaining to

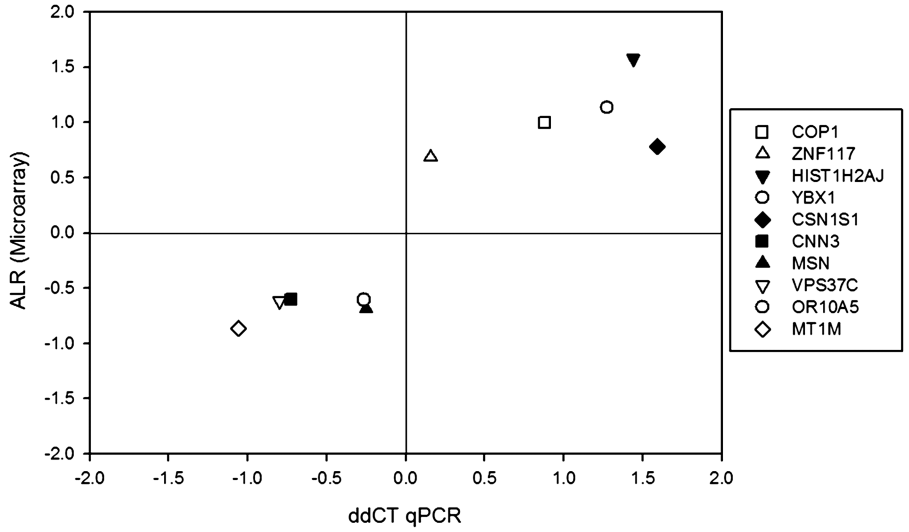

Figure 2 qPCR analysis of selected differentially-expressed mRNAs in MDD subjects and matched controls. The *x* axis denotes microarray-reported expression differences (ALR), whereas *y* axis shows qPCR-obtained differential expression values ($\Delta\Delta Ct$) for 10 selected transcripts. Each symbol represents a different gene, denoted in the figure legend. Note that all the observations occupy quadrants 2–3 (and no observations are located in quadrants 1 and 4), showing that all microarray-qPCR results were concordant in directionality of change. The ALR-$\Delta\Delta Ct$ results were highly correlated ($r = 0.92$; $P < 0.001$).

the cytokine pathway in the MDD group, with a nominal *P*-value = 0.0062 and FDR *q*-value = 0.03 (Table 3). Of the 18 constituents of this pathway, 13 were significant in the core enrichment of the dataset, including IL-1α, IL-2, IL-3, IL-5, IL-8, IL-9, IL-10, IL-12A, IL-13, IL-15, IL-18, interferon gamma (IFNγ), and lymphotoxin α (TNF superfamily, member 1; TNFβ).

Discussion

The microarray mRNA expression analysis in this study from 14 matched depressed and matched control brains tissue samples from BA10 suggest that there may be increased inflammatory or other pro-apoptotic stress in the MDD sample group. For example, there was significant up-regulation of caspase-1 dominant-negative inhibitor, pseudo-ICE (COP1). Caspases are cysteine proteases that are involved in cell death cycles through apoptosis or inflammation. COP1 has significant homology with the caspase recruitment domain of caspase-1 and it acts as an inhibitor of both caspases-1 and -4,[29] both of which are up-regulated in face of inflammatory stress.[30] Increased expression of COP1 is protective of caspase-mediated cell death.[31] Similarly, both FKSG2, a putative apoptosis inhibitor protein and YBX1, were significantly up-regulated in the MDD sample. Relatively, little is known about FKSG2, although it is known to be up-regulated by cysteinyl leukotrienes, which are mediators of tissue inflammation.[32] YBX1 is a member of the cold shock domain superfamily of proteins, a highly evolutionarily conserved transcription factor.[33] YBX1 is involved in a variety of intracellular functions, including transcriptional regulation (either activation or repression), DNA repair, and response to stress signals.[33] One function of YBX1 is to bind to free mRNA, which prevents translation ('mRNA masking') and degradation.[33] YBX1 is up-regulated in face of a variety of stressors, including hyperthermia, UV radiation, and viral infection, and it is involved in nuclear viral replication. Up-regulation of the expression of specific anti-apoptotic proteins suggests the possibility of apoptotic stress in this region, which has been hypothesized as a causal factor in depression.[34,35] In fact, a variety of studies have implicated impaired neuroplasticity, reduction in regional brain volumes, and decreased neuronal and glial cell body numbers in depressed patients relative to controls, all of which implicate apoptosis.[35]

Traditional gene expression analysis has tended to focus on single genes that are differentially expressed between two groups. However, although this is a conservative and highly rigorous approach, it may miss subtle, but important dysregulation of genes distributed across networks of related pathways.[27] GSEA was developed to analyze gene set data, and has been successfully applied to discover distributed networks of genes in a number of conditions, such as b-cell lymphoma[36] and prostate cancer.[37] The results of gene set analysis in this study amplify the evidence of up-regulation of the expression of genes involved in response to stress and inflammation. GSEA tests *a priori* gene groupings based on underlying biological similarities. In this analysis, only the pre-set 'cytokine' grouping showed significant up-regulation (Table 3). Although the absolute fold increase for each was modest, the set analysis suggests dysregulation of pro-inflammatory mechanisms. A number of cytokines were up-regulated, including IL-1α, IL-2, IL-3, IL-5, IL-8, IL-9, IL-10,

Table 3 Gene set enrichment analysis

(A) BioCarta pathways showing gene enrichment

BioCarta pathway name	*Size (genes)*	*Enrichment score*	P-*value*	Change direction
VEGFPATHWAY	25	0.657942	0.008163	Decreased
CDMACPATHWAY	15	0.699933	0.02268	Decreased
INTEGRINPATHWAY	33	0.549887	0.03527	Decreased
ECMPATHWAY	20	0.629207	0.038855	Decreased
PROTEASOMEPATHWAY	16	0.760643	0.041929	Decreased
ATMPATHWAY	18	0.529419	0.04918	Decreased
MCALPAINPATHWAY	22	0.62423	0.049587	Decreased
CYTOKINEPATHWAY	*18*	*−0.79414*	*0.006211*	Increased

(B) GSEA cytokine pathway constituents (listed by Running ES rank)

Gene symbol	*Gene name*	*Rank metric score*[a]	*Running ES*[b]	*Core enrichment*
IL-18	Interleukin 18 (interferon-γ-inducing factor)	−0.031	−0.7628	Yes
IL-5	Interleukin 5 (colony-stimulating factor, eosinophil)	−0.061	−0.7613	Yes
LTA	Lymphotoxin α (TNF superfamily, member 1; TNFβ)	−0.075	−0.7345	Yes
IFNG	Interferon, γ	−0.098	−0.7007	Yes
IL-15	Interleukin 15	−0.106	−0.6492	Yes
IL-12A	Interleukin 12a (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	−0.113	−0.5942	Yes
IL-1A	Interleukin 1, α	−0.114	−0.5334	Yes
IL-10	Interleukin 10	−0.131	−0.4741	Yes
IL-8	Interleukin 8	−0.137	−0.4033	Yes
IL-13	Interleukin 13	−0.167	−0.3277	Yes
IL-2	Interleukin 2	−0.208	−0.2289	Yes
IL-9	Interleukin 9	−0.228	−0.1106	Yes
IL-3	Interleukin 3 (colony-stimulating factor, multiple)	−0.245	0.0182	Yes
IL-6	Interleukin 6	0.074	−0.6347	No
IL-4	Interleukin 4	0.036	−0.6943	No
IL-12B	Interleukin 12b (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	0.016	−0.7183	No
IL-16	Interleukin 16 (lymphocyte chemoattractant factor)	0.007	−0.7297	No
TNF	Tumor necrosis factor (TNF super family, member 2 [TNFα])	−0.011	−0.7515	No

Abbreviations: ES, enrichment score; GSEA, Gene Set Enrichment Analysis.
[a]Rank metric: GSEA uses the signal-to-noise metric to rank the genes (for details, see 'Metrics for Ranking Genes' at http://www.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html?Run_GSEA_Page). The larger the signal-to-noise ratio, the larger the differences of the means (relative to the s.d.). A high signal-to-noise metric indicates greater separation between phenotypes.
[b]Running ES: This reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes (for details, see 'GSEA Statistics' at http://www.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html?Run_GSEA_Page).

IL-12A, IL-13, IL-15, IL-18 (also known as IFNγ-inducing factor), as well as IFNγ itself and lymphotoxin α (TNFβ), most of which are pro-inflammatory (Table 3). An exception is IL-10, which is an anti-inflammatory cytokine that inhibits synthesis of IL-2, IL-3, and IFNγ, which may be up-regulated in response to the increase in these cytokines. Together with the up-regulated expression of COP1, YBX1, and FKSG2, these results suggest a local environment experiencing significant inflammatory stress.

Evidence for inflammation as a basis for depression is supported by earlier research suggesting a connection between depression and pro-inflammatory cytokines and related proteins.[38,39] It has been postulated

that certain pro-inflammatory cytokines act on the central nervous system causing so-called 'sickness behavior' in both human beings and animals. Symptoms that are common during infection share features with depression, including down mood, loss of interest in usual activities (anhedonia), anorexia, sleep disturbance, and decreased locomotor activity.[38] In fact, therapeutically administered IFNs such as IFNα for hepatitis C can produce a syndrome that is indistinguishable from depression.[40] Moreover, this can be attenuated or prevented by treatment with anti-depressant medication.[41,42] Several anti-depressants have been shown to suppress pro-inflammatory cytokine production and to release endogenous anti-inflammatory cytokines such as IL-10.[43–47]

A variety of studies have found elevated peripheral cytokines and other pro-inflammatory mediators in depression, including C-reactive protein, IL-1, IL-6, TNFα, and IFNγ in MDD (for reviews see Raison *et al*.,[38] Dantzer,[48] Garcia-Bueno *et al*.,[49] and Miller *et al*.[50]). To our knowledge, however, this study is the first demonstration of elevated levels of cytokines in brain in MDD, which suggests that the elevations of cytokines showed in earlier studies are actually reflected in the brain itself. This strongly supports inflammation as a mediator of depression in at least some patients with depression.

Although immune activation is associated with peripheral immune cells such as neutrophils, natural killer cells, and macrophages, it is now clear that locally resident cell types including microglia, astrocytes, and neurons mount innate immune responses in the central nervous system.[51] As an example, acute immune activation of microglia, such as that seen with the Gram-negative endotoxin lipopolysaccharide, can result in a robust release of TNFα, IL-1α, IL-1β, and IL-6.[52] Similarly, stress may also activate similar immune responses in brain without the presence of typical pathogen-associated molecules.[53] However, both chronic immune activation[52] and chronic stress[54] leads to a progressive reduction in the release of these molecules and an increase in anti-inflammatory mediators such as IL-10.[55] Chronic immune activation may account, then, for the apparent discrepancy between the results we have shown in brain and the earlier observation in peripheral samples, in which increased IL-6 and TNFα have been most consistently shown.[38,50]

The actual causal pathway connecting pro-inflammatory cytokines and depression remains obscure, but there are some mechanisms that have been implicated. For example, inflammatory cytokines including IL-1β, TNFα[56] IL-3 and IL-5[57] activate p38 mitogen-activated protein kinase, which has been shown to induce catalytic activation of the serotonin transporter, which increases uptake of serotonin, decreasing synaptic availability.[56,58] Both IL-1β and TNFα increase serotonin uptake in both a rat embryonic raphe cell line and synaptosomes isolated from mouse brain.[56,58] In addition, pro-inflammatory cytokines, including IFNγ, activate the enzyme indoleamine 2,3-dioxygenase leading to the synthesis of kynurenine from tryptophan, the precursor to serotonin. This shift to kynurenine synthesis leads to a relative depletion of serotonin, which could induce depressive symptoms in vulnerable individuals.[59] The kynurenine pathway leads to the synthesis of several neuroactive products, including the neuroprotective intermediaries kynurenic acid and picolinic acid and the NMDA receptor agonist neurotoxin quinolinic acid.[60] Inflammatory cytokines also have significant effects on dopamine and associated reward-related behavior. For example, in rats, IL-2 (which was elevated in the tissue samples from depressed patients in this study) has been shown to decrease dopamine efflux in nucleus accumbens and to inhibit self-stimulation through an electrode in the median forebrain bundle, a model of rewarding behavior.

Although this study implicates local inflammatory and apoptotic stress in BA10, the mechanisms mediating these effects are unclear. One clue may be found in one of the genes shown to be reduced relative to controls in the present sample, MT1M. Metallothioneins (MTs) represent a family of cysteine-rich proteins that bind heavy metals through the cysteine-thiol group.[61,62] In particular, MT1M is involved in zinc homeostasis through zinc-thiol binding. Under normal conditions, zinc concentrations are tightly regulated by MT1M and related zinc-binding (thiolate) proteins.[61,62] MTs also directly regulate oxidative stress by cysteine capture of free radicals.[63] Under conditions of oxidative stress, MTs scavenge free radicals, releasing zinc into the cytoplasm. Reduced availability of MT1M would be expected to enhance oxidative stress directly by altered regulation of superoxide radicals, and indirectly by effects on zinc metabolism. Low MT1M, then, would create a state of vulnerability to oxidative stress by creating a redox imbalance by a failure to maintain an appropriate reducing environment. In fact, earlier research indicates that MTs exert anti-apoptotic functions through a variety of mechanisms, including inflammatory and oxidative stress.[61] Notably, a recent study has shown that exogenous administration of MT markedly attenuated microglia activation and quinolinic acid expression induced by IFNγ.[60] Clearly, this novel finding needs further investigation to determine what, if any, function MTs may have in depression vulnerability.

The results of this study are at variance with earlier post-mortem brain microarray analyses in MDD,[2,3,5,8,64] which could be related to a variety of factors, including differences in microarray platforms, brain region, and clinical phenotype. BA10 is involved in reward-related cognitive processing[12] and is activated in response to highly reinforcing stimuli.[13,14] Cellular dysfunction in BA10 may, then, account for some of the deficits in response to reward that are found in depression.[15,16] Only one earlier study investigated BA10, and found a dissimilar set of differentially expressed genes.[17] A variety of

factors may have contributed to these differences. For example, in that study, nine of 11 depressed subjects were on psychotropics, whereas the current set were free of known psychotropic medications. Another possible source of variance with this and other microarray studies may be the clinical phenotype chosen for our study. Most of the patient samples in this study came from persons with the clinical phenotype of melancholic MDD, which shows particularly severe deficits of reward responding;[65] the proportion of samples in earlier studies from the melancholic subtype is unknown. Melancholic MDD is a distinct clinical phenotype, which tends to be characterized by marked psychomotor disturbances, sleep and appetite changes, and reduced responses to hedonic stimuli.[65,66] Moreover, the melancholic subtype of depression may be more likely to show dysregulation of peripheral markers of immune function.[53] For example, one study showed that whereas non-melancholic depressed patients exhibited increased leukocytes (particularly natural killer cells), but no differences in cytokines, melancholics had elevated IL-2, IL-10, and IFNγ, which is consistent with the results of the gene set analysis in this study.[67] Whether melancholia is specifically characterized by local apoptotic and inflammatory stress in brain should be further investigated in future studies.

There are significant limitations to the current analysis. We have shown altered expression of specific genes and gene sets; however, this may or may not be reflected in actual gene products or downstream functions. Further, the results were limited to a single brain area; whether these differences are found in other brain regions is unknown. We also oversampled the melancholic subtype of MDD; the accuracy of this subtype distinction depends on the precision of the diagnosis made through interview with the next of kin, which may or may not reflect the actual diagnosis in life.

In summary, this study suggests that, in BA10, depressed persons show evidence of increased inflammatory and apoptotic stress, including elevations in specific cytokines as well as anti-apoptotic proteins. Although the causal mediators of these abnormalities are not known, oxidative stress is implicated. Clearly, further research is needed to validate these findings and to further investigate causal mechanisms.

Conflict of interest

The authors declare no conflict of interest.

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