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Inflammation and Neurological Disease-Related Genes are Differentially Expressed in Depressed Patients with Mood Disorders and Correlate with Morphometric and Functional Imaging Abnormalities

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Abstract

Depressed patients show evidence of both proinflammatory changes and neurophysiological abnormalities such as increased amygdala reactivity and volumetric decreases of the hippocampus and ventromedial prefrontal cortex (vmPFC). However, very little is known about the relationship between inflammation and neuroimaging abnormalities in mood disorders. A whole genome expression analysis of peripheral blood mononuclear cells yielded 12 protein-coding genes (ADM, APBB3, CD160, CFD, CITED2, CTSZ, IER5, NFKBIZ, NR4A2, NUCKS1, SERTAD1, TNF) that were differentially expressed between 29 unmedicated depressed patients with a mood disorder (8 bipolar disorder, 21 major depressive disorder) and 24 healthy controls (HCs). Several of these genes have been implicated in neurological disorders and/or apoptosis. Ingenuity Pathway Analysis yielded two genes networks, one centered around TNF with NFKB β , TGF β , and ERK as

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Conflict of Interest

Wayne Drevets, M.D. has consulted for Johnson & Johnson, Pfizer, Myriad/RBM and Eisai. The other authors have no financial disclosures to make.

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connecting hubs, and the second network indicating cell cycle and/or kinase signaling anomalies. fMRI scanning was conducted using a backward-masking task in which subjects were presented with emotionally-valenced faces. Compared with HCs, the depressed subjects displayed a greater hemodynamic response in the right amygdala, left hippocampus, and the ventromedial prefrontal cortex to masked sad versus happy faces. The mRNA levels of several genes were significantly correlated with the hemodynamic response of the amygdala, vmPFC and hippocampus to masked sad versus happy faces. Differentially-expressed transcripts were significantly correlated with thickness of the left subgenual ACC, and volume of the hippocampus and caudate. Our results raise the possibility that molecular-level immune dysfunction can be mapped onto macro-level neuroimaging abnormalities, potentially elucidating a mechanism by which inflammation leads to depression.

Background

Extant data indicate that people with mood disorders who are currently depressed display abnormalities of immune function including chronic activation of the innate immune system (Dantzer et al., 2008, Maes, 2008, Miller et al., 2009, Drexhage et al., 2010). These data take the form of (a) elevated levels of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 6 (IL6) in the serum or plasma of patients with major depressive disorder (MDD) (Maes, 1999, Howren et al., 2009, Dowlati et al., 2010), (b) the differential expression of immune-related genes in the monocytes of people with bipolar disorder (BD) (Padmos et al., 2008), (c) the anti-inflammatory effect of antidepressant medication (De Berardis et al., 2010), (d) the induction of depression in up to 40% of cancer and hepatitis C patients during treatment with interferon alpha (INF α) (Capuron and Miller, 2004), (e) the induction of depressive symptoms in healthy individuals administered endotoxin (Eisenberger et al., 2009) or typhoid vaccine (Harrison et al., 2009a), (f) the epidemiological association between mood disorders and diseases with an autoimmune or inflammatory component such as multiple sclerosis (MS), cardiovascular disease and diabetes mellitus (Dalton and Heinrichs, 2005, Van der Kooy et al., 2007, Nouwen et al., 2010), (g) the increased production of neurotoxic kynurenine metabolites such as quinolinic acid in depressed individuals (Dantzer et al., 2011), (h) the association of single nucleotide polymorphisms (SNPs) in the major histocompatibility complex (MHC) region with BD in genome-wide association studies (GWAS) (Williams et al., 2011, Hamshere et al., 2012) and (i) a reconceptualization of the immuno-privileged status of the CNS in light of literature demonstrating crosstalk between the brain and periphery via the vagus nerve, transport of cytokines across the blood brain barrier (Johnston and Webster, 2009), and brain infiltration by activated peripheral monocytes (D'Mello et al., 2009).

Patients with MDD and BD also display neuromorphometric and neurophysiological abnormalities – in particular, functional magnetic resonance imaging (fMRI) studies show that compared with healthy controls, depressed patients display altered hemodynamic responses of the medial prefrontal cortex-limbic network to emotionally-valenced face stimuli and that these neurophysiological abnormalities are associated with the negative emotional processing biases characteristic of depression (Savitz and Drevets, 2009b). For example, using the backward masking paradigm that is described in the methods section below, we previously demonstrated that compared with healthy controls, MDD patients show an increased hemodynamic response in the amygdala and hippocampus to masked sad versus masked happy faces whereas healthy controls showed an increased hemodynamic response in the amygdala to masked happy versus masked sad and neutral faces (Victor et al., 2010). Moreover, these processing biases were subsequently reversed in MDD subjects by antidepressant treatment with a selective serotonin reuptake inhibitor.

Regarding neuromorphometric abnormalities, our group first demonstrated that the mean gray matter volume of the subgenual anterior cingulate cortex (sgACC), in particular the left sgACC, is abnormally reduced in patients with MDD and BD (Drevets et al., 1997) and that this abnormality is associated with a reduction in glial cell number (Ongur et al., 1998). These findings have been independently replicated by multiple laboratories (reviewed in (Drevets et al., 2008) and (Savitz and Drevets, 2009a), and decreased volume of sgACC has been proposed to be a potential endophenotype of MDD (Savitz and Drevets, 2009b). A reduction in gray matter volume of the hippocampus (effect size of ~0.4) is another often replicated finding in MDD (see meta-analyses by (Videbech and Ravnkilde, 2004, Koolschijn et al., 2009, Kempton et al., 2011), while reductions in volume of the caudate are increasingly being reported in both MDD and BD (see meta-analyses of (Koolschijn et al., 2009, Yu et al., 2010, Bora et al., 2012).

Nevertheless, despite compelling evidence for dysfunction of both the medial prefrontal cortex (mPFC)-limbic network and the innate immune system in certain populations of people with mood disorders, very little is known about the relationship between molecular markers of inflammation and neurophysiological abnormalities in depression. This information is important because it may bring us closer to understanding the mechanisms behind the neurophysiological and/or neuroanatomical abnormalities observed in mood disorders.

Here we present results in which one index of immune function, namely the gene expression of peripheral blood mononuclear cells (PBMCs), was significantly correlated with a robust depression-related neurophysiological abnormality, the hemodynamic response of the mPFC-limbic network to masked, emotionally-valenced faces. A number of the genes that were differentially expressed in the PBMCs of our mood disorder group are associated with neurological disorders and/or apoptosis. Given these findings, we also present the results of a *post-hoc* analysis of the correlation between the mRNA expression levels of these genes and morphometric abnormalities of the left sgACC, hippocampus, and caudate.

Methods

Subjects

Subjects provided written informed consent after receiving a full explanation of the study procedures and risks, as approved by the University of Oklahoma Health Sciences Center IRB. We studied 8 adults with BD (4 BD type 1 and 4 BD type 2), 21 adults with MDD, and 24 healthy, unrelated controls (HC) (table 1). Both a structured (Structured Clinical Interview for the DSM-IV-TR) (First et al., 1996) and an unstructured (with a psychiatrist) psychiatric interview were obtained on all patients and healthy controls.

The mood disorder patients met DSM-IV criteria for recurrent primary MDD in a current major depressive episode or BD in a current major depressive episode, and had a Hamilton Depression Rating Scale (HDRS) score in the moderately-to-severely depressed range. These patients did not receive any psychotropic medications for at least 3 weeks (8 for fluoxetine) prior to the blood-draw and fMRI scanning. Treatment with medication was not discontinued for purposes of study participation. Patients either declined to be treated with medication or were diagnosed with a mood disorder for the first time prior to participation. Exclusion criteria were as follows: serious suicidal ideation or behavior, medical conditions or concomitant medications that were likely to influence cerebral blood flow or neurological function including cardiovascular, respiratory, endocrine and neurological diseases, a history of drug or alcohol abuse within 6 months or a history of drug or alcohol dependence within 1 year (DSM-IV criteria), current pregnancy or breastfeeding, and general MRI exclusion criteria such as magnetic implants or claustrophobia.

The healthy control individuals had no personal or family (first-degree relatives) history of psychiatric illness assessed using the Structured Clinical Interview for the DSM-IV-TR and the Family Interview for Genetic Studies (FIGS) (Maxwell, 1992).

Gene Expression Microarrays

Blood was obtained from the participants within 3 days of scanning. Whole blood was drawn into Becton Dickinson (BD) Vacutainer ethylene diamine tetraacetic acid (K₂EDTA) tubes (Franklin Lakes, NJ). PBMCs were isolated over a Ficoll-paque Plus (GE Healthcare Biosciences, Pittsburgh, PA) density gradient. PBMCs were frozen in fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored in liquid nitrogen.

Total RNA was isolated from frozen PBMCs using QIAshredder (QIAGEN, Valencia CA) columns for cell homogenization followed by use of the RNeasy Micro Kit (QIAGEN). DNA was eliminated through on-column DNase I (QIAGEN) treatment according to the manufacturer's directions. RNA was quantified spectrophotometrically. RNA integrity was assessed using capillary gel electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA) to determine the ratio of 28s:18s rRNA in each sample.

A total of 300 ng of total RNA from each sample was labeled using the Illumina Total Prep RNA Amplification Kit following manufacturer's directions (Ambion; Austin TX). cRNA was hybridized overnight to Illumina Human HT-12 v4 Expression BeadChips containing 47,231 probes (Illumina, Inc., San Diego, CA). Microarrays were washed to high stringency and labeled with streptavidin-Cy3 (Amersham Biosciences; Piscataway, NJ) prior to scanning on an Illumina iScan scanner. Signal intensities were collected using the Gene Expression Module (v 1.9.0) in the Genome Studio software (v 2011.1, Illumina).

Quantile normalization and log (base 2) transformation of the microarray data were performed using Matlab (R2007b) software (Mathworks, Inc). Data were analyzed with Biometric Research Branch (BRB)-Array Tools software developed by Richard Simmons and the BRB-Array Development Team (National Cancer Institute, NIH) after applying a log expression variation filter to screen out genes that are not likely to be informative based on the variance of each gene across the arrays. Statistically significantly differentially expressed genes were identified using a 5% false discovery rate.

Interactions between differentially expressed gene products were explored using Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, Redwood, CA). IPA attempts to map each differentially expressed gene provided within its curated database. Mapped genes are overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. No genes were added or forced into a given pathway by the authors. All networks retained for interpretation have a Fisher's Exact test p-value < 1 × 10⁻⁶.

rtPCR (real time polymerase chain reaction) Validation of Microarray Data

Microarray data were validated using real time relative rtPCR. cDNA was generated using the Qiagen Quantitect Whole Transcriptome Kit (Valencia, CA). All PCR validations were performed with Taqman Gene Expression Assays (Life Technologies, Carlsbad, CA) using Taqman Fast Advanced Master Mix on the Life Viia 7 Real Time PCR system (Life Technologies). DataAssist Software v. 3.01 (Life Technologies) was used to analyze the data. Since fold change numbers can vary significantly between microarray and rtPCR, directional change was used to compare the results obtained by microarray and rtPCR.

Backward Masking Task

Nineteen of the 24 healthy control subjects and 27 of the 29 subjects with current MDD (n=20) or BD (n=7) were scanned while performing a rapid event-related fMRI backward masking task. Prior to each of two nine-minute scans, subjects were shown two neutral target faces. They were instructed to remember the faces and indicate with a button press using their right thumb whether each face that appeared on the screen was a target or non-target face. Subjects were instructed that sad, happy and neutral faces would appear on the screen and they were to judge if a target face was present based on the person's identity, not the expression on their face. Subjects performed an abbreviated version of the task outside the scanner to demonstrate their understanding of the instructions.

Each two second face trial displayed sad, happy, or neutral faces in pairs of two consisting of a "masked" and "unmasked" face. The following stimulus pairs were presented: sad-neutral (SN), happy-neutral (HN), neutral-sad (NS), neutral-happy (NH) and neutral-neutral (NN) (figure 1). A sad or happy face that appeared in the masked face position was never also shown in the unmasked face position for any trial. Within a single trial, the identity of the masked face was never the same as the identity of the unmasked face, but the gender of the two faces was always the same. The gender for all stimulus pairings was balanced across runs.

Each two second face trial consisted of the presentation of a masked face for 26ms immediately followed by an unmasked face for 107ms and then 1866ms of baseline (no image). In both runs, the five stimulus types were presented 40 times each. Two-hundred 2-second face trials and sixty 2-second baseline trials were presented in addition to a baseline measurement at the beginning and end of the run. The interstimulus interval (baseline trials) varied between 0–8 seconds. The face and baseline trial presentation order was optimized for rapid event-related fMRI using optseq2. Stimuli were presented to the subjects in the scanner gantry using E-Prime software and visual display system hardware at 75Hz to allow for the short presentation times. The accuracy of the face stimulus timings was verified using a photodiode and oscilloscope. Faces were selected from the NimStim Set of Facial Expressions.

fMRI Data Acquisition

Functional and structural MRI scans were acquired on a 3T GE Discovery MR750 scanner with an 8-channel phased-array head coil. The fMRI was performed using a gradient-echo echoplanar imaging (EPI) pulse sequence (39 slices, TE=27ms, TR=2000ms, flip angle=90 degrees, 96 × 96 matrix, field of view=24 cm, voxel dimensions = 2.5 × 2.5 × 2.9 mm). A total of 274 fMRI images were obtained during each of two 9-minute runs while participants performed the task. Three images were discarded at the beginning of each run to allow for steady-state tissue magnetization. A high-resolution anatomical scan was obtained using a magnetization-prepared, rapid gradient echo (MP-RAGE) pulse sequence optimized for tissue contrast resolution (120 slices, TR=5 ms, TE=1.95 ms, flip angle=8 degrees, FOV=240 × 216 mm, voxel size= 0.94 × 0.94 × 1.1 mm, prep = 725 ms, delay=1400) to provide an anatomical framework for the functional imaging analyses.

fMRI data analysis

Data were analyzed using Analysis of Functional NeuroImages (AFNI, <http://afni.nimh.nih.gov/>) software (Cox and Hyde, 1997) within the framework of the General Linear Model (GLM) (Friston, 2005). Pre-processing of single-subject fMRI data included volume registration and slice timing correction, outlier filtering, motion correction, conversion to standardized brain space, intensity standardization, and spatial smoothing using Gaussian kernel with full width at half maximum (FWHM) of 6 mm. The following

regressors were included in the GLM model: 5 stimulus conditions (SN, HN, NN, NS, NH) and 6 motion parameters as nuisance covariates to account for potential artifacts due to head motion. The GLM model beta coefficients were computed using the AFNI program 3dDeconvolve. These beta coefficients were used for whole-brain statistical analysis between groups. A group t-test was performed using the AFNI program 3dMEMA to compare the hemodynamic response to SN vs. HN in MDD compared to HC subjects. The statistical threshold for detecting a difference between groups was set at $p < 0.05$, uncorrected, using a minimum cluster size of 20 voxels. The beta weights reported in the results were extracted from each of three functionally-defined regions of interest based on the *a priori* hypothesis of significant differences in the SN vs. HN contrast between groups in the amygdala, vmPFC and hippocampus.

Structural MRI

The morphometric scans were also acquired on the 3T GE Discovery MR750 scanner (GE Medical Systems) but with a 32 channel phased-array coil system (Nova Medical) using a magnetization-prepared, rapid gradient echo (MP-RAGE) pulse sequence optimized for tissue contrast resolution: (TR=5msec, TE=2.01 msec, FOV 240r192; voxel size=0.94 × 0.94 × 0.90 mm; prep=725 msec, delay=1400 msec, flip=8°). Data obtained with this sequence were available for 22 patients with a mood disorder (5 BD, 17 MDD) and 11 healthy controls. The automated segmentation program, FreeSurfer (<http://surfer.nmr.mgh.harvard.edu/>) was used to obtain gray matter volumes of the hippocampus and caudate, and cortical thickness measures of the left “subcallosal gyrus” (figure 2). “Subcallosal gyrus” in FreeSurfer corresponds closely to the posterior portion of the sgACC considered homologous with the infralimbic cortex (Brodmann Area [BA] 25).

Statistical Analysis

The normalized mRNA levels, beta-weights for the SN-HN contrast, and volume/thickness measurements were obtained for each individual. Samples from BD and MDD patients were pooled for purposes of analysis due to considerations of sample size ($n=29$). Pearson’s correlation analyses (2-tailed, $p < 0.05$) were conducted using Statistical Package for the Social Sciences (SPSS) (V17) in order to test for associations between PBMC gene expression and the neuroimaging data within the mood disorder group, only. An analysis of variance (2-tailed, $p < 0.05$) was used to test for differences between the mood disorder group and thickness of the left sgACC, and gray matter volume of the hippocampus and caudate.

Results

Gene Expression Profiles in PBMC of Mood Disorder Patients

Using a stringent 5% false discovery rate and a difference of at least 1.25 fold expression between the groups, we identified 26 genes that differed in expression between the mood disordered and healthy control group. These data are shown in detail in the supplementary table. Of the 26 differentially expressed genes, 12 encoded proteins with known functions: ADM, APBB3, CD160, CFD, CITED2, CTSZ, IER5, NFKBIZ, NR4A2, NUCKS1, SERTAD1, and TNF. Fourteen others are transcribed but produce no functional protein (italicized in the supplementary table). These include a 28s rRNA transcript and various small nuclear or nucleolar transcripts, plus six pseudogenes. What is most striking among the other genes is the extent to which many have been associated with neurological features or psychiatric illness (e.g. ADM, APBB3, CFD, CITED2, CTSZ, NR4A2, and TNF. See supplementary table). rtPCR was conducted on the 12 protein-coding genes in order to validate the microarray data. We found that only CTSZ, CFD, and NFKBIZ did not have a directional match when comparing the microarray analyses to the rtPCR analyses.

IPA software was used to map the differentially expressed genes from mood disordered versus healthy control samples. Two networks of interacting gene products were identified (figures 3 and 4). In these networks, red and green colored gene symbols indicate genes expressed to a significantly greater or lesser extent, respectively, in the mood disordered subjects versus healthy controls. In the first network (figure 3), numerous differentially expressed genes, including TNF, were linked to non-differentially expressed nuclear-factor kappa-B (NF κ B), transforming growth factor beta (TGF β), and extracellular signal-regulated kinase (ERK) which act to regulate inflammation. The probability of finding this number of differentially expressed genes in a group of randomly selected genes in a functional network of this size is $< 10^{-20}$. Cell cycle and/or kinase signaling anomalies are suggested in the second network (figure 4). The probability of finding this number of differentially expressed kinase signaling genes in a group of randomly selected genes in a functional network of this size is $< 10^{-6}$. A third network, containing only the differentially expressed gene IER5 is not shown. Not surprisingly, pseudogenes were not recognized by Ingenuity's knowledge base.

fMRI

Consistent with our previously reported findings in an independent sample (Victor et al., 2010), the mood disordered patients showed a greater hemodynamic response than healthy controls in response to masked sad versus masked happy faces (SN-HN) in the right amygdala (Talarach coordinates x, y, z = 22, -8, -15; cluster size = 49, $t=2.74$, $p<0.05$), the right ventromedial PFC (vmPFC) (x, y, z = 8, 48, -1; cluster size = 113, $t=3.07$, $p<0.05$), and the left hippocampus (x, y, z = -31, -20, -13; cluster size = 31, $t=3.10$, $p<0.05$). The regions of significant activation in the amygdala, vmPFC and hippocampus are shown in figure 5, and the mean beta-coefficients for the healthy control and mood disorder groups are shown in figure 6.

Within the mood disorder group significant correlations were found between right amygdala activation and gene expression of APBB3, CFD, NFKBIZ, and NUCKS1. Hemodynamic response of the right vmPFC correlated significantly with the gene expression of CFD, while hemodynamic response of the left hippocampus was significantly correlated with APBB3 and CFD expression. These data are displayed in table 2.

Morphometric MRI

There was no significant difference between the HC (n=11) and the mood disorder group (n=22) in the thickness of the left sgACC ($2.6 \pm 0.3\text{mm}$ versus $2.5 \pm 0.3\text{mm}$, $t=1$, d.f.=31, $p=0.342$), and the gray matter volume of the hippocampus ($8051.5 \pm 841.2\text{mm}^3$ versus $8227.2 \pm 846.2\text{mm}^3$, $t=0.6$, d.f. =31, $p=0.584$) or caudate ($7110.1 \pm 900.6\text{mm}^3$ versus $7125.9 \pm 1110.2\text{mm}^3$, $t=0.4$, d.f. = 31, $p=0.968$). Because this sample was small for a volumetric study, and because many of the genes that differed in expression between the mood disorder and HC groups were involved in neurological diseases, we conducted a *post-hoc* analysis of the relationship between the volumetric data and the microarray data. These results are shown in table 3. CD160 and CITED2 were associated with thickness of the left sgACC. Gray matter volume of the hippocampus was significantly correlated with gene expression of NR4A2, while ADM, NFKBIZ, NR4A2, SERTAD1, and TNF were correlated with gray matter volume of the caudate.

Discussion

Because it was not feasible to obtain brain tissue for gene expression analysis in this study, RNA from PBMCs was used as an alternative to search for depression-related gene expression differences that may exist in multiple tissues including the brain. Here we not only showed that gene expression differences existed in PBMCs from a cohort of depressed

patients relative to healthy, unrelated controls, but also that the expression patterns of many of these genes were associated with neurophysiological and neuromorphometric abnormalities in the patients with mood disorders.

These results are discussed in more detail in the supplementary section but some of the more interesting genes that were differentially expressed between the mood disorder and healthy control groups are briefly discussed here.

Adrenomedullin (ADM), which was increased in the mood disorder group versus the healthy control group, is a vasorelaxant that is part of the first-line protective (i.e. anti-inflammatory) response to toxic or aversive stimuli such as lipopolysaccharide (LPS) (Wong et al., 2005). A recent genome-wide association study (GWAS) implicated a single nucleotide polymorphism (SNP) in the vicinity of the ADM gene in a sample of subjects with type II BD (Huang et al., 2010). In addition, a functional SNP in the gene was associated with response to paroxetine (Glubb et al., 2010), and ADM was reported to be upregulated in a study using PBMCs in psychologically stressed individuals (Miller et al., 2008).

The expression of nuclear receptor subfamily 4, group A, member 2 (NR4A2, also known as NURR1) was increased in the mood disorder group versus the healthy control group. NR4A2 has neuroprotective effects and plays an important role in the differentiation and maintenance of dopaminergic neurons of the ventral tegmental area (VTA) during development. The mRNA expression of NR4A2 was found to be decreased in the prefrontal cortex (BA9 and BA46) of subjects with MDD and BD at *postmortem* (Xing et al., 2006). (Buervenich et al., 2000) sequenced the NR4A2 gene and reported that 2 patients with schizophrenia and one patient with BD, but no healthy controls, had missense mutations in the third exon of the gene that led to significant decreases in transcriptional activity. Similarly, (Chen et al., 2001) found an exonic deletion in two unrelated patients with schizophrenia that was not present in any healthy controls.

The expression of tumor necrosis factor α (TNF) was increased in the mood disorder group versus the healthy control group. Two recent meta-analyses reported a small but statistically significant increase in plasma levels of TNF in depression (Dowlati et al., 2010, Liu et al., 2012). In addition, a polymorphism in the TNF gene was associated with increased risk of developing MDD in the Genetic Association Information Network (GAIN) GWAS dataset (Bosker et al., 2011).

We chose whole genome expression of PBMCs as our marker of immune function for several reasons. Firstly, this approach is not constrained by our incomplete knowledge of the specific immunological pathways underpinning mood disorders and could therefore facilitate the detection of unknown immunological pathways that mediate depression-associated pathophysiology. Secondly, a number of studies have produced compelling results by using this approach. For example, (Padmos et al., 2008) performed whole-genome expression profiling on microarrays using purified monocytes and reported a distinct gene expression signature composed of aberrantly elevated expression of genes in patients with BD representing inflammatory and inflammation-related pathways. A follow-up rtPCR study using 19 of these genes showed >80% sensitivity and specificity for distinguishing unmedicated BD subjects and the depressed offspring of a BD parent from healthy controls (Padmos et al., 2008). Thirdly, alternative markers of inflammation such as plasma or serum levels of pro-inflammatory cytokines have certain limitations. Although meta-analyses have demonstrated that the levels of proinflammatory cytokines are elevated in depression, the effect size is very small (Dowlati et al., 2010, Liu et al., 2012), and in many individuals cytokine levels are below the detectable limit. An analysis that relied on correlating each

subject's cytokine levels with neuroimaging data would thus suffer from a significant loss of statistical power.

A small number of studies have examined the relationship between immune function and neuroimaging measures in healthy volunteers. (Marsland et al., 2008) reported an inverse correlation between IL-6 concentration and gray matter volume of the hippocampus. Although we did not obtain measurements of IL-6 in the plasma, we did observe a positive association between the expression of NR4A2 (table 3), which is neuroprotective, and hippocampal gray matter volume.

In grieving women, IL1 β concentration was associated with a greater blood oxygenation-dependent (BOLD) response in the sgACC during the recall of grief-related versus neutral words (O'Connor et al., 2009b). (Harrison et al., 2009a) conducted a double-blind, cross-over study in which patients received either a typhoid vaccination or placebo and then completed an implicit emotional face perception task. The inflammation induced by the typhoid vaccination was shown to result in an increased hemodynamic response in the sgACC during the emotional face processing task, and reduced connectivity between the sgACC and regions such as the ventromedial PFC, amygdala, and nucleus accumbens.

Here we found an inverse association between thickness of the left sgACC and the expression of CD160 (table 3). We also found a trend towards a significant inverse correlation between CITED2 expression and left sgACC thickness that may be explained by the fact that CD160 activation can trigger the production of cytokines while CITED2 is activated by LPS and by cytokines such as IL-2, IL-4, IL-6, and IFN- γ .

Using a similar design to (Harrison et al., 2009a), (Inagaki et al., 2012) showed that relative to the placebo condition, administration of low-dose endotoxin resulted in increased amygdala reactivity during the presentation of fearful faces. This finding is potentially consistent with our data showing correlations between hemodynamic response of the amygdala to sad faces and genes such as CFD and NFKBIZ which are involved in the inflammatory response.

(Eisenberger et al., 2010) demonstrated that endotoxin-induced anhedonia was associated with reduced hemodynamic response in the ventral striatum to monetary rewards. Although we were not able to measure the ventral striatum *per se* with FreeSurfer, we did observe significant correlations between gray matter volume of the caudate and gene expression of ADM, NFKBIZ, NR4A2, SERTAD1, and TNF.

The Ingenuity Pathway Analysis which showed an interaction between NF κ B and several of the differentially expressed genes (figure 3) is consistent with a genome-wide expression study of PBMCs in the familial care-givers of brain cancer patients (i.e. stressed individuals) which reported increased expression of transcripts with response elements for NF κ B (Miller et al., 2008). Similarly, increased NF κ B expression was recently reported in women with childhood abuse-related post-traumatic stress disorder (PTSD) (Pace et al., 2012).

The fact that CFD, IL23A, and NR4A2 have all been implicated in multiple sclerosis (MS) (see supplementary file) is consistent with microarray studies of *postmortem* brains which have reported overlapping functional pathways in BD and MS (reviewed in (Konradi et al., 2012)). The prevalence of BD in MS patients is double that of the general population (Schiffer et al., 1986), and white matter hyperintensities are present in a subpopulation of patients with BD, including pediatric cases (reviewed in (Savitz and Drevets, 2009a)). While the neuropathological process underlying MS may cause symptoms of BD, (Kosmidis et al., 2012) reported an extensive presence of both BD and MS in a multigenerational family, raising the possibility of a genetic overlap between the two disorders.

More generally, the fact that a significant number of the differentially expressed genes have been associated with various autoimmune diseases is intriguing given the growing awareness of the biological links between mood disorders and autoimmune thyroiditis (Vonk et al., 2007), pernicious anemia, Crohn's disease, autoimmune hepatitis (Eaton et al., 2010), and type I diabetes (Korczak et al., 2011).

It is also interesting that APBB3, CTSZ, NR4A2, and NUCKS1 have all been associated with various forms of dementia or aging-related pathology (see supplementary file). Depression is not only a prodromal symptom of AD, but early-onset, primary mood disorders increase the risk of developing AD later in life. A 17 year-follow-up of participants in the Framingham Heart study showed that depressed subjects had more than a 50% increase in the risk for developing AD and other dementias later in life (Saczynski et al., 2010). Similarly, the Baltimore Longitudinal Study of Aging showed a monotonic increase in the risk for all-cause dementia based on the number of episodes of depression in the 24 year-follow-up period (Dotson et al., 2010). (Leonard, 2007) has suggested that the inflammation-induced neuropathological changes associated with depression precipitates the neurodegenerative changes characteristic of AD and other dementias.

A significant strength of our study is that all participants were medication free. Mood stabilizing, antipsychotic, and antidepressant medications have all been demonstrated to affect immune function (Goldstein et al., 2009, De Berardis et al., 2010, Chen et al., 2012). Moreover, concurrent treatment with psychiatric medications confounds neuroimaging studies; for example antidepressant medication eliminates the hemodynamic response bias to masked sad faces (Victor et al., 2010) and lithium is neuroprotective, increasing gray matter volume (Moore et al., 2000, Bearden et al., 2007, Savitz et al., 2010).

We tested for correlations between the imaging data and microarray data within the mood disorder group alone, rather than in the full sample of mood disorder and healthy control subjects. We took this approach for two reasons. Firstly, we wished to avoid the so-called "double-dipping" confound (Kriegeskorte et al., 2009). That is, correlating these fMRI and microarray data in the combined healthy control and mood disorder samples that we already demonstrated to differ on these immunological and neuroimaging variables would be a circular analysis. Secondly, the relationship between inflammation and neuroimaging abnormalities may be attenuated in healthy controls since even if psychiatrically healthy individuals do suffer from inflammation, they are presumably able to compensate.

Our data raise the question of whether there is a causal relationship between depression-associated immunological dysfunction and the neuroimaging abnormalities observed in people with mood disorders. Clearly our study design does not allow us to answer this question but there are plausible mechanisms by which dysregulation of the immune system may lead to neuropathology and/or neurophysiological abnormalities. Firstly, activated microglia are able to initiate an inflammatory cascade of cytokines, chemokines, and reactive nitrogen and oxygen species which directly damage neurons and compromise the reuptake of glutamate by astrocytes, leading to a secondary wave of excitotoxicity (Miller et al., 2009). Secondly, pro-inflammatory cytokines have been shown to upregulate indoleamine 2,3 deoxygenase (IDO) leading to a reduction in peripheral tryptophan levels together with increased synthesis of neurotoxic kynurenine metabolites (Capuron et al., 2002, Wichers et al., 2005, O'Connor et al., 2009a, Dantzer et al., 2011). Thirdly, interferon α (IFN α), TNF, and IL1 β have been shown to upregulate the serotonin transporter (5-HTT) *in vitro* (Tsao et al., 2006, Zhu et al., 2006), while intraperitoneal administration of double-stranded DNA (which mimics a viral infection) increased expression of IFN α and 5-HTT but decreased extracellular serotonin in the medial prefrontal cortices of mice (Katafuchi et al., 2006). Fourthly, inflammation may lead to a decrease in tetrahydrobiopterin (BH $_4$)

which is a cofactor for the production of both serotonin and dopamine (van Amsterdam and Opperhuizen, 1999, Hoekstra et al., 2006).

Limitations

It is not clear what cell types were responsible for the mood disorder-associated differences in gene expression in our study. The fact that we examined PBMCs rather than monocytes may explain why we were unable to replicate the results of (Padmos et al., 2008). Signal-to-noise ratio may be increased in future studies by focusing on one particular type of immune cell.

We combined the BD and MDD patient groups together. While it is possible that MDD and BD are etiologically distinct disorders that display distinct transcriptomic and neuroimaging signatures, there is extensive genetic, clinical, neurophysiological, and neuromorphometric overlap between the two disorders that is not captured by current nosological categories. The failure of current diagnostic categories to align with emerging findings from clinical neuroscience and genetics has culminated in the Research Domain Criteria (RDOC) initiative of the National Institutes of Health which promotes a neuroscience-based approach to psychiatric classification that entails discarding extant nosology (Insel et al., 2010). It is within the spirit of this paradigm that we combined the MDD and BD groups in this study. Moreover, given the challenges in recruiting unmedicated patients with BD, merging the BD and MDD group had the practical utility of increasing the statistical power of the study.

No independent marker of general inflammation - for example C-reactive protein (CRP) - was available. It would have been informative to assess whether CRP levels differed between the healthy control and mood disorder groups, and whether CRP levels correlated with differential gene expression within the mood disorder group.

We examined peripheral immune function (PBMC expression) rather than the gene expression of microglial cells, and thus it is theoretically possible that our data do not reflect central immune function. However, there are a number of reasons why PBMC expression might provide an accurate account of central immune function. In general, the expression levels of many biological processes have been shown to be comparable between whole blood and the prefrontal cortex (Sullivan et al., 2006). Secondly, as discussed above, there is extensive evidence for crosstalk between the CNS and periphery. Pathogen-associated molecular patterns (PAMPS) and cytokines activate primary afferent nerves such as the vagus nerve which projects to numerous brain regions involved in the behavioral and autonomic responses to stress such as the periventricular nucleus of the hypothalamus, periaqueductal gray matter, and bed nucleus of the stria terminalis (BNST) (Dantzer et al., 2008). Moreover, cytokines and certain neurotoxic kynurenine metabolites such as 3-hydroxykynurenine freely diffuse across the blood brain barrier (Schwarcz et al., 2012) while activated macrophage cells are capable of infiltrating the CNS (D'Mello et al., 2009). Thirdly, experimental manipulations of the peripheral immune response using endotoxin or the typhoid vaccine produce an increase in symptoms of depression concomitant with abnormal brain activity in regions including the sgACC, dorsolateral PFC, amygdala, ventral striatum, and insula (Harrison et al., 2009a, Harrison et al., 2009b, Eisenberger et al., 2010, Inagaki et al., 2012). Fourthly, 7 out of the 12 genes (ADM, APBB3, CFD, CITED2, CTSZ, NR4A2, and TNF) that were differentially expressed in the mood disorder group, have been associated with neurological or psychiatric diseases, suggesting at least a partial correspondence between markers of peripheral and central immune function.

While the fMRI study was likely to have been adequately powered - with a moderate to large effect size of 0.7–0.8 (the required sample size for 80% power in an fMRI study designed using methods like those we applied is 20–26 subjects per group) (Kraemer and

Kupfer, 2006), the morphometric MRI analysis was underpowered, largely due to the relatively small subset of the control subjects for whom the volumetric MRI data were available, possibly explaining the absence of significant differences in volume/thickness between the subject groups. Given the dearth of whole-genome microarray studies in mood disorders, the adequacy of our sample size for the immunological component of the study is unclear. However, given the heterogeneity of patients with mood disorders at both the neurophysiological and immunological level, future studies would be enhanced by the inclusion of a greater number of participants.

Conclusion

In this study we attempted to bridge the gap between molecular-level abnormalities in immune function and macro-level neurophysiology and neuroanatomy. Our results provide initial support to the hypothesis that depression-associated immune dysfunction can be mapped onto depression-related neuromorphometric and neurophysiological abnormalities, and suggest that this approach may be a fruitful avenue for future research. The relationship between immunological and neurophysiological abnormalities is important to understand because it may allow for the identification of specific molecular pathways that mediate the neuroimaging abnormalities associated with mood disorders. Conceivably this knowledge will facilitate the development of new treatments for mood disorders. Clearly given the complexity of both immunological function and neuropathophysiology, the imprecision inherent in microarray and neuroimaging measurements, and the heterogeneous nature of mood disorders, our results are not straightforward to interpret and may have been confounded by both type I and type II errors. Larger replication samples may be needed to enhance the signal-to-noise ratio in this type of data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Depression-associated gene expression of mononuclear cells correlate with neuroimaging abnormalities, supporting the role of immune dysfunction in mood disorders

Figure 1.

Backward masking task design. Example images of sad and happy faces in the masked face position are shown for the sad-neutral (SN) and happy-neutral (HN) conditions with placeholders (“N”) to represent the presentation of neutral faces. The first face was presented for 26ms, followed by a 107ms neutral face, followed by no image for the remainder of the two-second trial. The interstimulus interval (ISI) ranged from 0–8 seconds. No image was displayed during the ISI.

Figure 2. Examples of FreeSurfer-derived masks for the thickness of the left posterior subgenual anterior cingulate cortex (top), hippocampus (middle), and caudate (bottom). The masks are shaded in red and shown in the sagittal plane.

Figure 3.

Ingenuity Pathway Analysis showing a network of known functional connections between 10 of the protein-coding genes that were differentially expressed in the mood disorder group. The red color indicates that the gene was overexpressed in the mood disorder group compared with the healthy control group, and the green color indicates that the gene was underexpressed in the mood disorder group relative to the healthy control group. Lines indicate known interactions between gene products.

Figure 4. Ingenuity Pathway Analysis showing a network of known functional connections between 3 of the protein-coding genes which were differentially expressed in the mood disorder group. Refer to the figure 2 legend for an explanation of the format.

Figure 5.

Image sections from a statistical parametric map of voxel t-values showing regions where the hemodynamic response to masked happy versus masked sad faces is significantly decreased (blue color) in the mood disorder group versus the healthy control group. Stereotaxic coordinates provided beneath each section image denote the distance in mm from the stereotaxic origin (anterior commissure), with positive x indicating right of midline (for sagittal sections), positive y indicating anterior (for coronal sections), and positive z indicating dorsal (for horizontal sections).

Figure 6.

Bar-histograms showing the beta-weights for the blood oxygen level dependence (BOLD) responses of the ventromedial prefrontal cortex, amygdala, and hippocampus for the SN-HN contrast. The beta-weights for the healthy control group are shown in yellow and the beta-weights for the mood disorder group are shown in blue. The negative beta-weights indicate a greater hemodynamic response to masked happy faces than masked sad faces, and the positive beta-weights indicate a greater hemodynamic response to masked sad than masked happy faces. The error bars represent the standard error of the mean.

Table 1

Subject Information

	Mood Disorder	Healthy Control	Statistic, p-value
Number	29	24	-
Age	35±10	34±12	T=0.34, p=0.736
Sex (% M)	32	52	X ² =2.1, p=0.148
BMI	28±5	26±5	T=1.7, p=0.097
HAM-D	25±7 range 6–39	0.4±0.7 range 0–2	T=14.5, p<0.001

BMI = body mass index, HAM-D = Hamilton Depression Rating Scale. Note that two BD subjects had scores below the moderate-to-severe range (scores of 6 and 13, respectively). The range of Ham-D scores in the mood disorder group without these 2 participants was 19–39.

Table 2

Pearson Correlations Between mRNA Expression of the 13 Significant Genes and Hemodynamic Response to Masked Sad versus Happy Faces (SN-HN) in the Mood Disorder Group (N=27).

Gene	Amygdala	vmPFC	Hippocampus
ADM	$r=-0.06$, $p=0.763$	$r=-0.06$, $p=0.775$	$r=-0.03$, $p=0.884$
APBB3	$r=0.40$, $p=0.037^*$	$r=0.10$, $p=0.625$	$r=0.40$, $p=0.038^*$
CD160	$r=-0.10$, $p=0.964$	$r=0.10$, $p=0.618$	$r=-0.05$, $p=0.821$
CFD	$r=-0.46$, $p=0.017^*$	$r=-0.46$, $p=0.016^*$	$r=-0.50$, $p=0.009^{**}$
CITED2	$r=-0.06$, $p=0.771$	$r=0.08$, $p=0.681$	$r=-0.02$, $p=0.928$
CTS2	$r=-0.18$, $p=0.234$	$r=-0.08$, $p=0.699$	$r=-0.06$, $p=0.780$
IER5	$r=0.22$, $p=0.272$	$r=-0.10$, $p=0.609$	$r=-0.13$, $p=0.511$
NFKBIZ	$r=0.44$, $p=0.022^*$	$r=0.16$, $p=0.423$	$r=0.24$, $p=0.225$
NR4A2	$r=0.27$, $p=0.179$	$r=0.14$, $p=0.476$	$r=-0.01$, $p=0.979$
NUCKS1	$r=-0.46$, $p=0.016^*$	$r=-0.26$, $p=0.184$	$r=-0.09$, $p=0.655$
SERTAD1	$r=0.20$, $p=0.331$	$r=-0.06$, $p=0.786$	$r=-0.05$, $p=0.799$
TNF	$r=0.07$, $p=0.731$	$r=-0.03$, $p=0.870$	$r=-0.05$, $p=0.793$

*
 $p<0.05$;

**
 $p<0.01$;

vmPFC = ventromedial prefrontal cortex

Table 3

Pearson Correlations Between mRNA Expression of the 13 Significant Genes and Morphometric Data in the Mood Disorder Group (n=22).

Gene	Left sgACC	Hippocampus	Caudate
ADM	$r=-0.25$, $p=0.254$	$r=0.15$, $p=0.531$	$r=0.46$, $p=0.031$ *
APBB3	$r=0.12$, $p=0.595$	$r=-0.08$, $p=0.726$	$r=0.24$, $p=0.284$
CD160	$r=-0.49$, $p=0.020$ *	$r=0.04$, $p=0.864$	$r=0.32$, $p=0.460$
CFD	$r=0.08$, $p=0.730$	$r=0.42$, $p=0.067$	$r=-0.23$, $p=0.297$
CITED2	$r=-0.42$, $p=0.054$	$r=0.01$, $p=0.993$	$r=0.38$, $p=0.083$
CTSZ	$r=-0.15$, $p=0.496$	$r=0.15$, $p=0.521$	$r=-0.10$, $p=0.644$
IER5	$r=-0.23$, $p=0.301$	$r=-0.11$, $p=0.639$	$r=0.33$, $p=0.134$
NFKBIZ	$r=0.00$, $p=0.999$	$r=0.09$, $p=0.718$	$r=0.48$, $p=0.024$ *
NR4A2	$r=-0.36$, $p=0.096$	$r=0.37$, $p=0.041$ *	$r=0.56$, $p=0.006$ **
NUCKS1	$r=0.38$, $p=0.081$	$r=-0.05$, $p=0.846$	$r=0.09$, $p=0.518$
SERTAD1	$r=-0.28$, $p=0.211$	$r=0.02$, $p=0.940$	$r=0.53$, $p=0.011$ *
TNF	$r=-0.12$, $p=0.609$	$r=0.09$, $p=0.707$	$r=0.43$, $p=0.047$ *

* $p<0.05$;

** $p<0.01$;

sgACC = subgenual anterior cingulate cortex