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Altered neuro-inflammatory gene expression in hippocampus in major depressive disorder

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Abstract

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Contributors: Craig A. Stockmeier, Gouri J. Mahajan, Eric J. Vallender, Michael R. Garrett, and Hamed Benghuzzi designed the study. Craig A. Stockmeier, with assistance from James C. Overholser, George Jurjus, and Lesa Dieter, wrote the research protocol for human subjects. Lesa Dieter interviewed decedents' families for retrospective psychiatric diagnosis, and she participated with James C. Overholser, George Jurjus, and Craig A. Stockmeier in assigning diagnoses based on the interviews. Craig A. Stockmeier and Gouri J. Mahajan identified subjects to be examined. Gouri J. Mahajan prepared tissues for RNA sequencing and qPCR, and together with Damien G. Romero and Maryam Syed performed the qPCR. Michael R. Garrett supervised RNA quality assessments and RNA sequencing. Gouri J. Mahajan performed the bioinformatic analyses, with assistance from Eric J. Vallender and Lavanya Challagundla. Statistical analyses and graphic preparations were performed by Lavanya Challagundla, Gouri J. Mahajan, and Eric J. Vallender. Gouri J. Mahajan wrote the first draft of the manuscript and all authors contributed to the editing of this manuscript and have approved its final form.

Ethical Statement

The material described in this manuscript is original research, has not been previously published (except in the form of an abstract) and has not been submitted for publication elsewhere while under consideration. The authors declare no conflict of interest, and all named authors have agreed to its submission and approved of the manuscript. If the paper is accepted it will not subsequently be published in the same or similar form in any language without the consent of the copyright-holder.

All work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed written consent was obtained from legally-defined next-of-kin for tissue collection, medical records, and interviews.

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Major Depressive Disorder (MDD) is a common psychiatric disorder for which available medications are often not effective. The high prevalence of MDD and modest response to existing therapies compels efforts to better understand and treat the disorder. Decreased hippocampal volume with increasing duration of depression suggests altered gene expression or even a decrease in neurogenesis. Tissue punches from the dentate gyrus were collected postmortem from 23 subjects with MDD and 23 psychiatrically-normal control subjects. Total RNA was isolated and whole transcriptome paired-end RNA-sequencing was performed using an Illumina NextSeq 500. For each sample, raw RNA-seq reads were aligned to the Ensembl GRCh38 human reference genome. Analysis revealed 30 genes differentially expressed in MDD compared to controls (FDR<0.05). Down-regulated genes included several with inflammatory function (*ISG15*, *IFI44L*, *IFI6*, *NR4A1/Nur-77*) and *GABBR1* while up-regulated genes included several with cytokine function (*CCL2/MCP-1*), inhibitors of angiogenesis (*ADM*, *ADAMTS9*), and the *KANSL1* gene, a histone acetyltransferase. Similar analyses of specific subsets of MDD subjects (suicide vs. non-suicide, single vs. multiple episodes) yielded similar, though not identical, results. Enrichment analysis identified an over-representation of inflammatory and neurogenesis-related (ERK/MAPK) signaling pathways significantly altered in the hippocampal dentate gyrus in MDD. Together, these data implicate neuro-inflammation as playing a crucial role in MDD. These findings support continued efforts to identify adjunctive approaches towards the treatment of MDD with drugs including anti-inflammatory and neuroprotective properties.

Keywords

depression; hippocampus; dentate gyrus; RNA-sequencing; neuro-inflammation

1. Introduction

Major depressive disorder (MDD), a common but serious disorder with a lifetime prevalence of 17 percent among the adult US population, is predicted to be the second leading cause of illness by 2020 (Kessler et al., 2003, Lecrubier, 2001). The economic burden of depression in the U.S., along with comorbid conditions, is estimated at over \$170B, and depressive disorders are the leading contributors to global burden of disease (Greenberg et al., 2015, Whiteford et al., 2013). Full remission from depression, using medications that target serotonin or norepinephrine, is achieved by less than 50 percent of patients (Berton and Nestler, 2006, Rush et al., 2006). The high prevalence of MDD and modest response to existing therapies compels efforts to better understand and treat the disorder.

1.1. Peripheral inflammation and depression

An association between inflammation and depression has been proposed following examination of both blood and brain (Barnes et al., 2017, Köhler et al., 2017, Mechawar and Savitz, 2016, Wohleb et al., 2016). A recent meta-analysis by Köhler and colleagues reported elevated peripheral levels of cytokines and chemokines in subjects with MDD as compared to control subjects (Köhler et al., 2017). Microglial activation in brain, leading to release of pro-inflammatory cytokines and chemokines, has implicated neuro-inflammation in the etiopathology of depression (Mechawar and Savitz, 2016). However, peripheral inflammation may not reflect inflammation in the brain as depressed subjects with

neuroimaging evidence for activated microglia in the brain do not demonstrate a significant correlation between peripheral and central markers of inflammation (Setiawan et al., 2015). In clinical studies, there is evidence for immune dysregulation and microglial activation in depression, while in pre-clinical studies repeated exposure of rodents to stress enhances brain levels of inflammatory markers and alters neuronal activity and plasticity (Wohleb, 2016).

1.2. Neuro-inflammation and depression

The role for neuro-inflammation in depression has been supported by some studies using postmortem prefrontal cortex. In microarray studies, an increase in expression of genes for tumor necrosis factor family receptor (*TNFRSF11B*), interferon $\alpha 6$ (*IFNA6*), interferon receptor 1 (*IFNAR1*), as well as *GATA1*, a transcriptional repressor which binds to the promoter for interleukin-6 (*IL-6*), an inflammatory cytokine, was detected in dorsolateral prefrontal cortex (Brodmann's area 9) of 15 pairs of subjects with MDD and matched control subjects (Kang et al., 2007, Kang et al., 2012). Additionally, there was a significant up-regulation of gene expression for several pro- and anti-inflammatory cytokines in prefrontal cortex (Brodmann's area 10) in 14 pairs of subjects with MDD and matched control subjects (Shelton et al., 2011). Levels of kynurenine pathway metabolites and cytokines, including interferon- γ and tumor necrosis factor (TNF)- α , were significantly decreased in ventrolateral prefrontal cortex in subjects that experienced a depressive disorder (Clark et al., 2016). In prefrontal cortex of teenage suicide victims, the expression of protein and mRNA for the pro-inflammatory cytokines interleukin (*IL*)- 1β , *IL-6* and *TNF- α* was significantly increased (Pandey et al., 2012). A later study done by the same group reported significant increases in toll-like receptor (*TLR3* and *TLR4*) mRNA and protein in prefrontal cortex in depressed suicide victims (Pandey et al., 2014). Activation of toll-like receptors leads to the release of pro-inflammatory cytokines and suggests an innate immune response to neuronal injury (Pandey et al., 2014). Torres-Platas and colleagues examined white matter of the anterior cingulate cortex and reported elevated mRNA expression for pro-inflammatory cytokines, including ionized calcium binding adaptor molecule 1 (*IBA1*), leukocyte common antigen (*CD45*), and monocyte chemotactic protein-1 (*MCP-1* or *CCL2*) in suicide victims with a depressive disorder, as compared to normal control subjects (Torres-Platas et al., 2014).

Recent next-generation sequencing studies have examined whole-transcriptome expression in postmortem brain tissue. In MDD and suicide, Pantazatos and colleagues reported lower expression of transcripts involved in immune-related cellular function, and functional deficits in oligodendrocytes, astrocytes, microglia, and endothelial cells (Pantazatos et al., 2017). In a recent elegant study, Labonté et al. demonstrated significant differences in the inflammatory transcriptome between males and females with MDD in several brain regions, including the subiculum, a part of the hippocampal formation (Labonté et al., 2017). While current studies linking neuro-inflammation to depression in postmortem human brain are correlative, a causal relationship in humans has yet to be elucidated.

1.3. Neuro-inflammation and the hippocampus in depression

Cellular and molecular pathology involving synaptic function and neuro-inflammation has also been identified in the hippocampus in MDD. There is a significant reduction in the volume of the hippocampus, as well as increased packing density of neurons in CA1 subregion, with increasing duration of depression (Cobb et al., 2013, Sheline et al., 1996). Decreased numbers of hippocampal granule cells and a diminished volume of the granule cell layer were also noted in the hippocampus in MDD (Boldrini et al., 2014, Boldrini et al., 2013). Microarray gene profiling of the hippocampal dentate gyrus has revealed altered expression of genes related to synaptic transmission in both MDD and in a rodent model of depression using chronic unpredictable stress (Datson et al., 2012, Duric et al., 2010, Duric et al., 2013). RNA-sequencing of the hippocampus revealed differential expression of synaptic-related genes and immune/inflammation-related co-expression modules associated with MDD (Kim et al., 2016). A recent neuroimaging study reported a significant elevation in translocator protein density in several brain regions, including prefrontal cortex and hippocampus, in antidepressant drug-free patients with MDD experiencing a depressive episode (Setiawan et al., 2015). Holmes et al. noted a significant elevation in translocator protein density in the anterior cingulate cortex of nine patients with MDD and suicidal thoughts, but not five without suicidal thoughts (Holmes et al., 2017). Hannestad et al. reported no change in translocator protein density in a variety of brain regions of ten patients with mild to moderate depression (Hannestad et al., 2013). Translocator protein is associated with microglia, the immune-competent cells of the brain which can release cytokines, and its expression is enhanced during microglial activation in the course of neuro-inflammation.

1.4. Hypothesis

Postmortem human tissue samples from depressed and/or suicide victims provide a strong foundation for evidence-based exploration of hypothesis-driven research. In this study, we performed next-generation sequencing to examine the entire transcriptome of the hippocampal dentate gyrus in subjects with MDD. In exploratory analyses, we also subcategorized depressed subjects based on single vs. multiple episodes of depression, and those dying by suicide or natural causes. Based on published studies in human brain and in rodent models of chronic stress, we hypothesized that there would be differential expression of genes related to neuroplasticity and neuro-inflammation in depression.

2. Methods and material

2.1. Human subjects

Brain tissues were collected at autopsy at the Cuyahoga County Medical Examiner's Office (Cleveland, OH) and frozen as described (Duric et al., 2010). Cause of death was determined by the Medical Examiner (Supplementary Table S1). Recruitment, tissue collection, and retrospective psychiatric assessment were performed under a protocol approved by the Institutional Review Boards at University of Mississippi Medical Center and University Hospitals Cleveland Medical Center, Cleveland, OH. All work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed written consent was obtained from legally-defined next-of-kin for tissue collection, medical records, and interviews. Evidence of neuropathological or neurological disorders in

any subjects was grounds for exclusion. The medical examiner's office evaluated blood and urine samples from all subjects for psychotropic medications and substances of abuse.

2.2. Psychiatric assessment

A master's level social worker administered the Structured Clinical Interview for DSM-IV Axis I Disorders (First et al., 2002) to knowledgeable informants for all subjects. Psychopathology was independently assessed by a board certified clinical psychologist and a board certified psychiatrist and a consensus diagnosis was reached in conference using information from interview and medical records. DeJong and Overholser demonstrated a high degree of agreement between diagnoses of MDD when interviewing next-of-kin vs. diagnoses based on interviewing patients (DeJong and Overholser, 2009).

Subjects were selected that met diagnostic criteria for MDD according to Diagnostic and Statistical Manual of Mental Disorders IV (American Psychiatric Association, 1994). Depressed subjects met criteria for a major depressive episode within the last two weeks of life, except one in full and three in partial remission. Control subjects did not meet criteria for a lifetime or current Axis I psychiatric disorder (Supplementary Table S1). Comorbid disorders in the subjects with MDD included: anxiety disorder not otherwise specified (NOS) (2), generalized anxiety disorder (3), secondary dysthymic disorder (2), and pathological gambling and delusional disorder (1). Only one depressed subject met criteria for a current psychoactive substance use disorder – sedative, hypnotic, anxiolytic use disorder NOS. Three MDD subjects had an alcohol abuse disorder in remission, and one MDD subject had a prior history of cannabis dependence. Codeine plus cyclobenzaprine were present postmortem in one psychiatrically-normal control subject. Among the depressed subjects, the following psychoactive compounds were present postmortem: ethanol (3), codeine (1), oxycodone (1), and amitriptyline, oxycodone plus lorazepam (1). Additional clinical details on subjects with MDD are included in Supplementary Table S2. Medical records of the subjects were examined for the presence of an inflammatory disease in the months prior to death. In control subjects, two individuals had a medical history at the time of death that was consistent with inflammation – asthma and urinary tract infection. Among subjects with MDD, one individual had a diagnosis of arthritis.

2.3. Tissue preparation

Tissue samples were selected from 23 subjects with MDD (14 male, 9 female) and 23 psychiatrically-normal control subjects (14 male, 9 female) pair-matched for age and sex (Supplementary Table S1). Tissues were provided by the Postmortem Brain Core at the University of Mississippi Medical Center. Of the subjects with MDD, 17 died of suicide while 6 died of natural causes, and 17 subjects experienced chronic or multiple episodes of depression, while 6 experienced a single episode of depression. There were no statistically significant differences between the depressed and control subjects in age, postmortem interval, tissue pH, or RNA quality index (RQI). RQI values are reported vs. RNA integrity number (RIN) as both are highly comparable but RQI demonstrates somewhat better reproducibility and sensitivity than RIN (Riedmaier et al., 2010).

The anterior body of the hippocampus was dissected from the right temporal lobe, rapidly frozen in 2-methylbutane (Fisher Scientific, Asheville, NC, USA) cooled by dry ice, and stored at -80°C . A cryostat was used to section frozen tissues ($60\text{ }\mu\text{m}$). An adjacent section for each subject was processed for Nissl staining with cresyl violet and the location of the dentate gyrus was identified using a Nikon E600 microscope (2x objective) with Stereo Investigator (version 11.03.1, MBF Bioscience, Williston, VT, USA) (Cobb et al., 2013). A biopsy punch (2 mm; Sklar Instruments, VWR, Radnor, PA, USA), centered on the granule cell layer, was used to isolate the dentate gyrus in frozen sections (Supplementary Figure S1). About 25 punches were collected per subject from 8–10 sections, weighing about 20 mg. For all tissue processing and RNA isolation, each subject diagnosed with MDD was coded and yoked with a coded control subject matched for age and sex. Laboratory and bioinformatic personnel were blinded to individual demographics and diagnoses throughout tissue processing and data analyses.

2.4. RNA isolation and sequencing

Total RNA was isolated from tissue samples using the Invitrogen PureLink RNA Mini kit with Trizol (Life Technologies; Carlsbad, CA, USA) following manufacturer protocol. Quality control of total RNA was assessed using the Bio-Rad Experion Bioanalyzer for quality and Qubit Fluorometer for concentration measures. The RQI was 6.6 ± 2.1 (mean $\pm$ SD). Up to $1\text{ }\mu\text{g}$ of total RNA was used for gene expression analysis. Libraries were prepared using the TruSeq Stranded Total RNA LT Sample Prep Kit from Illumina (San Diego, CA, USA) per manufacturer protocol. Libraries were index-tagged and pooled for multiplexing at 16 samples per run. Next generation sequencing was performed on the Illumina NextSeq 500 platform using a paired-end read ($2 \times 101\text{ bp}$) protocol with the Illumina 300 cycle High-Output reagent kit. RNA isolation, library preparation, and next generation sequencing was performed by the Molecular and Genomics Core Facility at the University of Mississippi Medical Center, as described previously (Jung et al., 2017).

2.5. Read quantification and differential expression analyses

Differential expression of transcripts (i.e. mRNA and non-coding RNA) was assessed between subjects with MDD and controls. For each sample, raw RNA-seq reads (adapter-trimmed fastq files) were aligned to the Ensembl (version 76) GRCh38 human reference genome using GSNAP (Genomic Short-read-Nucleotide Alignment Program) (Wu and Nacu, 2010). Read quantification was performed on a cloud-based platform (Lumenogix, Inc., Santa Fe, NM, USA) using two algorithms; cufflinks, which produces values normalized by transcript length (Trapnell et al., 2012), and HTSeq-count, which calculates raw, hit count expression values (Anders et al., 2015). Although RQI values varied across samples, all samples produced roughly equivalent read counts and all samples confidently mapped the vast majority of reads to the reference genome (Supplementary Table S3). Neither read number, nor alignment rate was statistically correlated with RQI.

DeSeq2 (version 1.16.1) (Love et al., 2014) was used on the HTSeq (version 0.6.0) raw gene expression quantification data to analyze the impact of potentially confounding factors. This methodology allows for consideration of the covariates (age, postmortem interval, tissue pH, BMI (body mass index) and RQI in its design, using a negative binomial generalized linear

model to test for differential expression. These data are presented in box plot format using ggplot2 (version 2.2.1.9) in R.

Following read quantification, Lumenogix was used to perform differential expression analysis of genes and transcripts using cuffdiff (version 2.2.1) with Ensembl gene annotation. Differential expression analysis was carried out using all 46 subjects and reported as a heat map (CummeRbund version 2.18 in R) (Goff et al., 2013), and then secondary analyses were performed for all controls vs. 1) MDD-single episode (n=6), 2) MDD-multiple episodes (n=17), 3) MDD-suicide (n=17), and 4) MDD-non-suicide (n=6). Sex differences in gene expression associated with MDD were also explored by comparing control males vs. MDD-males (n=14) and control females vs. MDD-females (n=9). Cuffdiff performs intra-condition library scaling and then secondary scaling between conditions, also accounting for changes in isoform levels by maximum likelihood estimation of abundance (Trapnell et al., 2012). Expression of single-isoform variance is calculated using related negative binomial distribution and calculation of multiple-isoform variance is done by mixture of negative binomials with beta distribution parameters as mixture weights (Rapaport et al., 2013). Cuffdiff applies a correction for multiple testing and reports single isoform gene significance values (p-value) and false discovery rate corrected significance values (q-value). A stringent cut-off ($q < 0.05$) was used to identify differentially expressed genes for subsequent analyses.

2.6 Exploratory pathway analyses in control and MDD subjects

To identify relevant biological processes, molecular networks, signaling and metabolic pathways pathologically disrupted in MDD, the list of genes differentially expressed between the two cohorts was analyzed using Ingenuity Pathway Analysis (<https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>). All genes associated with a canonical pathway in Ingenuity's Knowledge Base were considered for the analysis. The cutoff for the Benjamini-Hochberg test of $q\text{-value} < 0.05$ was used for all the analyses. Canonical Pathway Analysis was then performed to identify the pathways from the Ingenuity Pathway Analysis library of canonical pathways for expression data that were statistically significant between cohorts. The significance of the association between the dataset and the canonical pathway was calculated by right-tailed Fisher's exact test. The significance denotes the probability that the association between genes from the uploaded dataset and canonical pathway-specific genes is due to random chance alone.

2.7. qPCR analysis

The same RNA used for RNA-seq analyses was subsequently used for qPCR validation. Total RNA (0.5 μg) was used as a template for synthesis of cDNA in a total reaction volume of 20 μl using Invitrogen SuperScript™ III Reverse Transcriptase. Five transcripts identified as differentially expressed in RNA-seq studies were chosen for validation: *ISG15*, *IFI44L*, *IFI6*, *NR4A1/Nur-77*, and *EDN1*. *GAPDH* and *ACTB* were used as reference genes. qPCR primer sequences were retrieved from the online PrimerBank database (Spandidos et al., 2010) and specificity was confirmed by the generation of a single band upon high resolution electrophoresis and melting curve analysis (data not shown). qPCR was performed in 96 well plates using the Bio-Rad CFX 96 real-time PCR detection system and SYBR Select

Master Mix for CFX (Applied Biosystems, Life Technologies Carlsbad, CA, USA). PCR reactions contained 10 μ l of the SYBR Green PCR mix, 0.04 μ l of 100 μ M forward and reverse primers, 1 μ l of cDNA, in a final volume of 20 μ l using nuclease free water. For all primer pairs, PCR cycling conditions were 50°C for 2 min and 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 1 min. PCR product quantification was performed by the relative quantification method (Clark et al., 2016, Livak and Schmittgen, 2001) and expressed as arbitrary units standardized against *GAPDH* (Schmittgen and Livak, 2008). These data are presented in graphic version using ggplot2 (version 2.2.1.9) in R. *ACTB* was also used as a second reference gene for validation.

3. Results

3.1. Demographics and read alignment

For each sample, 37 to 112 million reads were obtained and 64–96% were mapped successfully to the reference human genome, with most samples mapping above 85% (Supplementary Table S3). There were no significant associations between the number of reads obtained and PMI, tissue pH, or RQI. Similarly, no significant differences in alignment success were observed relative to any of these factors. There were no statistically significant differences between the depressed and control subjects in age ($t=0.05$, $p=0.96$), postmortem interval ($t=-0.23$, $p=0.82$), BMI ($t=0.28$, $p=0.78$), tissue pH ($t=-0.37$, $p=0.71$), or subsequent RQI ($t=-0.35$, $p=0.73$) (Supplementary Figure S2). DeSeq2 was used on the HTSeq raw gene expression quantification data to analyze the impact of potentially confounding factors on differential gene expression. When using age, PMI, BMI, tissue pH, and RQI as covariates in DeSeq2 multifactor analysis, there was no significant effect of these variables on the primary dependent variable.

3.2. Comparison of expression of transcripts between all MDD and all control subjects

Analysis of all control and MDD samples identified more than 600,000 unique transcripts of which 30 were identified as significantly differentially expressed following correction for multiple testing (FDR corrected q -value = 0.05; Table 1). Among the differentially expressed genes associated with MDD were several with inflammatory function (*ISG15*, *IFI44L*, *IFI6*, *NR4A1/Nur-77*), cytokine function (*CCL2/MCP1*), a GABA receptor (*GABBR1*), genes involved in angiogenesis (*ADM*, *ADAMTS9*), and a histone acetyltransferase (*KANSL1*) (Figure 1). When examining all control and MDD subjects, Ingenuity Canonical Pathway Analysis revealed a significant over-representation for genes related to interferon, chemokine and interleukin signaling as well as immune system related, TREM-1 signaling, and transcription factor related NR4A1/Nur-77 signaling (Figure 2). Gene expression and functional pathway analyses in depression were not altered by removing the one depressed subject taking amitriptyline or the one subject taking codeine (data not shown).

From these results, five genes were selected for validation using qPCR. Highly-expressed genes were selected in both cohorts, including one that was up-regulated and four that were down-regulated (Supplementary Table S4). Because of a limited amount of RNA for some samples, qPCR was used to analyze a subset of 35 samples from the same subjects also used for RNA-sequencing for which mRNA samples remained. For these five genes, there was a

significant differential change in gene expression in depression, as noted with RNA-seq. Using qPCR, all results were similar to RNA-seq in direction of changes in expression in MDD, however these results did not reach statistical significance (Supplementary Figure S3). Using either *GAPDH* or *ACTB* as a reference gene for qPCR yielded highly comparable results (data not shown for *ACTB*).

3.3. Exploratory subset analyses

We next compared whether observed patterns in differential gene expression was affected by the number of major depressive episodes. In depressed subjects with a single episode vs. control subjects, a total of 28 genes were differentially expressed: including inflammation-related *ISG1*, transcription related (*FOS*), dual specificity phosphatase related to cellular stress and cellular proliferation (*DUSP1*), neuroprotective and anti-apoptotic (*MTRNR2L1* and *MTRNR2L12*), metabolism-related (*RPL7A*) and major histocompatibility complex related (*HLA-DRB1*) (Supplementary Table S5). In depressed subjects with multiple episodes vs. control subjects, a total of 31 genes were differentially expressed: interferon-related *IFI44L*, cell adhesion related (*AJUBA*), inflammation-related chemokine ligand motif 2 (*CCL2*), suppressor of cytokine signaling (*SOCS3*), endothelin-related (*EDNI*), neuroprotective and anti-apoptotic (*MTRNR2L1*), and histone acetylation-related (*KANSL1*) (Supplementary Table S6).

In subjects with a single depressive episode, there was over-representation for genes related to extracellular signal-regulated kinases/mitogen-activated protein kinase (*ERK/MAPK*), *TNF-R1/2*, glucocorticoid receptor signaling, and retinoic acid receptor activation pathways (Supplementary Figure S4), while in subjects with multiple depressive episodes thioredoxin and the *IL-6*, *IL-17*, and *IL-22* signaling pathways were over-represented (Supplementary Figure S5).

In MDD-suicide vs control subjects, a total of 26 genes were differentially expressed: including a non-protein coding transcript (*KCNQ1OT1*), immune system-related (*C4A*) and chromatin regulation-related (*RBMX*), interleukin related (*IL32*) and non-protein coding transcripts such as *ADAMTS9* and *ADAMTS9-AS1* (Supplementary Table S7).

In MDD-non-suicide vs. control subjects, a total of 43 genes were differentially expressed: including glucocorticoid receptor signaling and immune system-related (*HSPA1A*), angiogenesis-related (*PTGSI*), and cytokinesis-involved (*MPLKIP*), immune system related (*CRISPLD2*, *CD93*) and transcription related (*NR4A1/Nur-77*) (Supplementary Table S8).

In MDD-suicide, there was over-representation for genes related to *IL-6*, *IL-9*, *IL-10*, and *IL-22* inflammatory pathways, interferon signaling, and JAK/STAT signaling pathways (Supplementary Figure S6). In MDD-non-suicide, the STAT3 pathway affecting phosphoinositide metabolism and eNOS signaling pathways were over-represented (Supplementary Figure S7).

Comparing MDD-males vs. male control subjects, a total of 44 genes were differentially expressed: including cytokinesis-involved (*MPLKIP*); transcription factor and neuronal

plasticity related (*NPAS4*), Early growth response protein 1 (*EGR1/Zif268*), metabolism-related (*RPL7A*) and immune system-related (*IL-32*) (Supplementary Table S9).

Comparing MDD-females vs. female control subjects, a total of 37 genes were differentially expressed including, calcium ion binding related (*EFCAB4B*) and inflammation-related (*C4A*), metabolism and immune system-related (*PNP* and *HLA-A*) (Supplementary Table S10).

In MDD-males, there was over-representation for genes related to glucocorticoid, iNOS signaling pathways, and IL-17A pathways (Supplementary Figure S8) while in MDD-females, cytokine production, Nur-77, and interferon signaling pathways were over-represented (Supplementary Figure S9).

Thus, in exploratory analyses for both gene expression and functional pathway analyses, comparing single vs. multiple episodes, or depressed subjects dying by suicide or natural causes, or depressed males vs. females, there were some unique, differentially expressed genes in each subset analysis but there was strong overlap with the more generalized analysis which included all 23 pairs of control and depressed subjects.

4. Discussion

The aim of this study was to determine the existence and nature of differences in gene expression in the hippocampus associated with MDD. The advantage of next generation sequencing is to explore the entirety of the transcriptome without *a priori* biases. The primary finding from this dataset, as well as in exploratory subsequent subset analyses, was an up-regulation of genes related to neuro-inflammation in MDD.

4.1. Differential gene expression in depression

The up-regulation noted in the dentate gyrus in chemokine and interleukin-related transcripts, such as *MCP-1/CCL2*, *IL-32*, *CD84*, *ADAMTS9*, and *HILPDA* is consistent with other reports of neuro-immune changes in brain in MDD (Kim et al., 2016, Labonté et al., 2017, Noto et al., 2014, Wohleb et al., 2016). In contrast, Pantazatos et al. (2017) reported decreased expression of chemokine receptors and ligands (*CCL2*, *CCL4*) and the pro-inflammatory cytokine (*IL8/CXCL8*) in dorsolateral prefrontal cortex in MDD (Pantazatos et al., 2017). In the current study, other down-regulated genes in depression with innate immunity-related functions include *ISG15*, *IFI44L*, and *IFI6*, an apoptosis-related gene, and *NR4A1/Nur-77*, a transcription factor activity-related gene. The present study also reports changes in regulators of angiogenesis, including adrenomedullin (*ADM*) and a disintegrin and metalloproteinase with thrombospondin motifs 9 (*ADAMTS9*), and in *KANSL1*, a histone acetyltransferase. ADAMTS proteoglycanases are also involved in neural repair and synaptic plasticity (Lemarchant et al., 2013). In the present study, of all the differentially expressed genes identified in the dentate gyrus in MDD, only *ISG15* was also noted by Labonté et al. as differentially expressed in the subiculum in MDD (Labonté et al., 2017).

4.2. Functional genomic analysis in depression

To further understand the mechanism underlying the disorder, functional genomic analyses were performed on the differentially expressed gene list in the primary dataset of 23 control and 23 MDD subjects. It revealed over-representation in MDD of signaling pathways for inflammation and interferon, cytokine and chemokine signaling, and ERK/MAPK signaling. In Labonté et al., functional groups of genes differentially expressed in females with depression include ERK/MAPK activity, inflammatory response and synaptic transmission (Labonté et al., 2017). Pantazatos et al. noted decreased expression of functional pathways in cell types including neurons, microglia, oligodendrocytes, astrocytes or endothelial cells, as related to chemokine receptors and ligands, and interleukins and pro-inflammatory cytokines (Pantazatos et al., 2017). The expression of one inflammation-related gene, *GREM1*, was down-regulated in an RNA sequencing study of the whole hippocampus in MDD (Kim et al., 2016). Thus, the current study has confirmed the involvement of neuro-inflammation in a large number of genes and functional pathways in MDD, and extended the observation to include a hippocampal subregion, the dentate gyrus, vital to neurogenesis and functional connectivity within the limbic system. The specific cell types affected in MDD in the current study cannot be resolved since the tissue dissections of the dentate gyrus included neurons and all types of glial cells, including microglia, astrocytes, and oligodendrocytes.

Evidence for microglial activation during major depressive episodes emerged in a neuroimaging study demonstrating elevated translocator protein density in the prefrontal cortex, anterior cingulate gyrus, and hippocampus (Setiawan et al., 2015). Stress-induced dysregulation of molecular and cellular pathways leads to microglial activation and altered neuroplasticity in MDD, as well as the initiation of inflammation as a homeostatic response (Wohleb, 2016). These results support the hypothesis that the neuron-microglia relationship and adaptive neuronal function play a major role in depression. This hypothesis is supported by observations that functional alterations in microglia in response to chronic stress is associated with diminished neuroplasticity and neurogenesis leading to depression-like behaviors (Lucassen et al., 2017, Yirmiya et al., 2015).

4.3. Exploratory analysis of subsets of depressed subjects

There was clinical heterogeneity associated within the MDD cohort in this study. Common functional pathways were over-represented in different subsets such as subjects with MDD experiencing a single depressive episode vs. those experiencing multiple depressive episodes, depressed subjects dying by suicide vs. natural causes, and male vs. females with depression. However, while there were some unique pathways affected in individual subsets, the overall picture emerging from canonical pathway analysis still revealed significant over-representation of neuro-inflammation-related pathways. For example, we saw many unique genes differentially expressed in dentate gyrus in males vs. female subjects with depression, although the same types of functional pathways were affected in both genders. In contrast, in subiculum and prefrontal cortical regions, Labonté et al. noted that the gene transcriptional patterns in males vs. females with depression were highly unique (Labonté et al., 2017). The results presented from the exploratory analyses of specific subsets of depressed subjects,

however, are only preliminary and need replication since the subsets are statistically underpowered.

4.4. Context of reported changes in gene expression in depression

A substantial literature supports a connection between inflammation, particularly the role of cytokines, and depression. A detailed review by Slavich and colleagues (Slavich and Irwin, 2014) proposed a multilevel social signal transduction theory of depression which hypothesizes that social stress triggers plasma pro-inflammatory cytokines leading to neuro-inflammation, resulting in various symptoms of depression. The accumulation of cytokines can have direct neurotoxic effects resulting in structural and functional changes in the brain regions crucial to processing emotions, leaving the brain vulnerable to depression (Kim and Won, 2017). Several studies report correlations between MDD and increased levels of peripheral or cerebrospinal fluid (CSF) cytokines such as IL-1 β , IL-6, and TNF- α (Goshen et al., 2008, Lindqvist et al., 2009, Maes et al., 2012, McAfoose and Baune, 2009, Miller et al., 2009). Higher blood levels of IL-8, IL-1 β and TNF- α in females, as compared to males, highlights a sex-specific relationship between inflammation and depression (Birur et al., 2017). Davami et al. reported significant higher level of IL-17 in 41 patients with MDD vs 40 healthy controls. These enhanced serum levels of IL-17 agree with our finding of a significant involvement of the IL-17 pathways in brain and support a connection between pro-inflammatory cytokines and depression (Davami et al., 2016).

The association of genes with depression has been examined in several genome-wide association studies (GWAS). The involvement of genes in MDD has been difficult to demonstrate due to factors such as heterogeneity of depressive symptoms, smaller sample sizes, and developmental and environmental influences and interactions (Barnes et al., 2017).

In a recent review, polymorphisms of inflammatory genes and related pathways, including *TNF- α* , *IL-1 β* , and interferon have been identified in MDD (Barnes et al., 2017). While the sample size in the present study is relatively small, functional genomic analysis also identified the involvement of similar inflammatory pathways in MDD.

4.5. Potential mechanisms whereby neuro-inflammation affects depression

The current study, along with other studies focusing on different brain regions, has strongly linked neuro-inflammation and depression, although neuro-inflammation as a cause of depression has not been firmly established in humans. Mechawar and Savitz addressed potential mechanisms whereby neuro-inflammation, through microglia, astrocytes, and oligodendrocytes, may cause behavioral changes associated with depression suggesting a neuroimmune etiology for a subset of patients (Mechawar and Savitz, 2016). Rodent studies by Banasr and Duman have demonstrated that reducing the astrocyte population in medial prefrontal cortex resulted in depression-like behaviors (Banasr and Duman, 2008) and the density of GFAP-immunoreactive astrocytes is decreased in prefrontal cortex and the hippocampal dentate gyrus in human subjects with MDD (Cobb et al., 2016, Miguel-Hidalgo et al., 2000). In rodents, LPS-induced central inflammation activates hippocampal astrocytes and microglia, and is followed by apoptosis of astrocytes, oligodendrocytes, and

neurons due to glutamate toxicity (Sharma et al., 2016). LPS-induced elevation of cytokines, including IL-6 and IL-1 β , has also been shown to decrease proliferation of NG2 cells, oligodendrocyte precursors, in the rodent hippocampus (Wennstrom et al., 2014). Finally, there are reductions in density of oligodendrocytes and changes in expression of oligodendrocyte-related genes in humans with MDD (Hayashi et al., 2011, Miguel-Hidalgo et al., 2017, Rajkowska et al., 2015). Thus, neuro-inflammation is closely tied to depression and pathology of glial cells.

A pathological mechanism whereby neuro-inflammation results in depression may involve the excitatory neurotransmitter glutamate and glial cells (Haroon et al., 2017). As part of the tripartite synapse, astrocytes play a crucial role in reuptake of synaptic glutamate. Activated microglia release L-glutamate which down-regulates the glutamate transporter (excitatory amino acid transporter 1, EAAT1) found on astrocytes (Takaki et al., 2012, Tilleux and Hermans, 2008). These enhanced levels of glutamate can be excitotoxic for neurons and glial cells (Dantzer and Walker, 2014). Evidence for an astrocytic pathology implicating glutamate in depression includes reduced expression of the astrocytic gap junction protein Cx43, astrocytic EAAT1 and 2 in orbitofrontal cortex and hippocampus, and glutamate-related genes in hippocampus in MDD (Duric et al., 2013, Medina et al., 2013, Medina et al., 2016, Miguel-Hidalgo et al., 2010, Miguel-Hidalgo et al., 2014). Thus, neuro-inflammation-related microglial activation may enhance synaptic levels of excitotoxic glutamate, suppress astrocytic function and density, and thereby impair hippocampal connectivity with limbic cortex, resulting in depression.

4.6. Neuro-inflammation in other psychiatric disorders

Neuro-inflammation has been increasingly identified in psychiatric disorders in addition to MDD. There are links between inflammatory markers and schizophrenia as noted by increased serum levels of IL-2, IL-6, IL-10, and IFN- γ (Balótšev et al., 2017), and IFITM1, IFITM2, IFITM3, APOL1, ADORA2A, IGFBP4 and CD163 (Hwang et al., 2013). In an RNA-seq study in subjects with schizophrenia, Fillman et al. observed a significant change in levels of cytokines and immune modulators (*IL-6*, *IL-8*, *IL-1 β* , and *SERPINA3*) of the dorsolateral prefrontal cortex (Fillman et al., 2013). Another recent report, using RNA sequencing in postmortem orbitofrontal cortex and hippocampus, noted enrichment of gene sets related to cytokine signaling in orbitofrontal cortex in schizophrenia, bipolar disorder, and MDD, while cytokine signaling was also affected in hippocampus in schizophrenia and bipolar disorder. However, the differential expression across the enriched gene sets related to cytokine signaling between the disorders was not consistent between brain regions or diagnostic groups (Darby et al., 2016). The evidence for involvement of neuro-inflammation in schizophrenia, bipolar disorder, MDD, however does not reveal whether neuro-inflammation is an underlying cause or a result of these disorders.

4.7. Neuro-inflammation in animal models

A role for neuro-inflammation in depression is supported by animal models. A neuro-inflammatory state induced in rodents by lipopolysaccharide (LPS) results in behaviors related to depression, an inhibition in hippocampal neurogenesis, and the expression of pro-inflammatory cytokines in hippocampus (Tang et al., 2016). Using a chronic mild stress

model of depression in mice, Goshen and colleagues (Goshen et al., 2008) demonstrated a causal relationship between IL-1 levels in hippocampus, decreased neurogenesis, and depression-like behavioral symptoms. In rats, the effects of chronic stress, treatment with LPS, and fluoxetine treatment on behavior were evaluated. LPS-induced inflammation prevented the antidepressant effects of fluoxetine and LPS increased blood levels of IL- β , thereby suggesting that underlying inflammation may play a role in resistance to antidepressant treatment (Wang et al., 2011). Modeling depression with chronic unpredictable stress in rodents revealed increased expression of pro-inflammatory cytokines such as IL- β , IL-6, and TNF- α , suggesting that peripheral markers of stress may influence neuro-inflammation and depression (Kubera et al., 2011).

4.8. Anti-inflammatory agents in treatment of depression

Rigorous clinical trials have examined the use of an anti-inflammatory agent as an adjunct to antidepressant medication in treating bipolar disorder and treatment-resistant depression (Dean et al., 2017, Husain et al., 2017, Soczynska et al., 2017). The chronic unpredictable mild stress model of depression in mice revealed that iptakalim, an opener of ATP-sensitive potassium channels, regulates neuro-inflammation and may have antidepressant properties (Lu et al., 2014). A meta-analysis of clinical trials reports that prescribing non-steroidal anti-inflammatory drugs and cytokine inhibitors as a part of antidepressant treatment may have therapeutic value in subtypes of depression and early stages of schizophrenia (Köhler et al., 2014, Müller, 2017). The present study strongly supports the involvement of neuro-inflammation in MDD, and may eventually lead to developing new antidepressant drug therapies.

4.9. Limitations and future investigations

There are several limitations to the current study. The number of subjects is relatively small, particularly for the subgroups of depressives, and statistically underpowered to use analysis of covariance to definitively assess the impact of death by suicide, single vs. multiple episodes, and male vs. female depressives. However, there was sufficient statistical power to reach conclusions on differential gene expression in all 23 pairs of control vs. depressed subjects. Another limitation is that the punches taken from the dentate gyrus also contained immediately adjacent hilus, in addition to the granule cell layer. The use of laser capture microdissection to sample only the granule cell layer and/or subgranular zone would allow selective assessment of the neurogenic region in depression. While the direction of change for each gene with qPCR was in the same direction as with RNA-seq, these values were not statistically significant. This was due in part to a smaller sample size because of limited amount of available RNA and greater variability in the qPCR methodology. While tissue pH, a marker of the agonal state, is an important determinant of RNA degradation in postmortem brain tissue, the samples collected here fell within published values and there was no significant relationship between tissue pH and gene expression (Kohen et al., 2014). Another potential limitation is that informant-based interviews were used for assessing psychopathology; however, DeJong and Overholser determined that an informant-based interview is a reliable proxy for directly interviewing the patient (Dejong and Overholser, 2009). For the results presented here to be replicated, it will be important to examine the same hippocampal sub-region using RNA-seq from subjects with MDD that lacked a

psychoactive substance use disorder at the time of death and were not taking a psychotropic medication.

In summary, this study provides the first solid support for altered neuro-inflammation based on changes in expression of a number of genes in the hippocampal dentate gyrus in well-characterized subjects with MDD. A significant strength of the study was careful control for the presence of any psychotropic drug or of any psychoactive substance use disorder. Regarding whether neuro-inflammation is causative for depression, altered expression of neuro-inflammation-related genes may reflect microglial activation that enhances synaptic levels of excitotoxic glutamate, suppresses astrocytic function and density, and thereby impairs hippocampal connectivity with limbic cortex, resulting in depression. Of particular interest will be to determine whether changes in DNA methylation or histone modification accompany the altered gene expression in MDD. It will also be of interest to determine whether the expression of miRNAs, in particular the ones targeting the differentially expressed genes reported in this study, are significantly altered in tissue samples taken from the same tissue blocks. It remains to be determined if anti-inflammatory drugs affecting the functional pathways identified here will be effective adjuncts to antidepressant medications. Assessing gene expression in animal models related to depression will be key to determining whether the transcripts reported here as altered in depression also play a critical role in the development of depression. Ultimately, if the changes in transcript expression noted in MDD can be modeled in chronic unpredictable stress and social defeat or other animal models, then pharmacological, molecular, and behavioral techniques could be developed to normalize these changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- In MDD, 30 genes are differentially expressed in dentate gyrus compared to controls
- MDD is associated with changes in genes with neuro-inflammatory and angiogenesis functions
- Inflammatory and neurogenesis-related signaling pathways are altered in MDD
- Anti-inflammatory treatments may be an adjunctive therapeutic approach to MDD

Figure 1.

The top 30 differentially expressed genes in the hippocampal dentate gyrus in 23 control and 23 MDD subjects identified by cuffdiff. The color scale for the heat map indicates the $\log_{10}(\text{FPKM}+1)$ gene expression values. FPKM: Fragments per kilobase of exon per million fragments mapped.

Figure 2.

The results of Ingenuity Pathway Analysis for statistically significant Canonical Pathways of the 30 differentially expressed genes in 23 control and MDD subjects as predicted using a Z-score algorithm. The y-axis displays the significant canonical pathways for the dataset that are involved in the analysis. The x-axis displays the $-\log$ of p-value as calculated by right-tailed Fisher's exact test. $-\log$ of p-value (1.3) is applied as the cut-off by default whereby pathways with a p-value equal to or less than 0.05 are displayed. Longer bar lengths are equivalent to greater statistical significance. Ratio indicates the number of molecules in a given pathway that meet cutoff criteria, divided by total number of molecules that make up that pathway and that are in the reference set.

Table 1

Differentially expressed genes in Control vs. MDD (N=23 subjects per cohort). The Benjamini-Hochberg procedure was used to compute the Q-value to control the false discovery rate (FDR) by correcting for multiple hypothesis testing.

		Mean Normalized Counts (FDR = 0.05)				
Ensembl Gene I.D.	Gene Symbol	Control	MDD	log2/sqrt	P-value (%)	Q-value (%)
ENSG00000187608	ISG15	21.61	10.23	-1.08	5.00E-05	0.034
ENSG00000137959	IFI44L	16.33	8.54	-0.93	5.00E-05	0.034
ENSG00000126709	IFI6	83.79	44.42	-0.92	5.00E-05	0.034
ENSG00000264286, ENSG00000066294	AL138930.1, CD84	2.37	5.44	1.20	5.00E-05	0.034
ENSG00000220997, ENSG00000241621, ENSG00000276347, ENSG00000107951	AL161651.1, GOLGA2P6, MIR7162, MTPAP	17.04	8.80	-0.95	5.00E-05	0.034
ENSG00000226937, ENSG00000277757, ENSG000000232554	CEP164P1, RP11-285G1.15, RSU1P2	1.07	0.48	-1.14	5.00E-05	0.034
ENSG00000148926	ADM	2.52	5.42	1.11	5.00E-05	0.034
ENSG00000187479, ENSG00000254409	C11orf96, RP11-613D13.4	3.85	8.26	1.10	5.00E-05	0.034
ENSG00000123358	NR4A1	8.04	4.15	-0.95	5.00E-05	0.034
ENSG00000130038, ENSG00000222338	EFCAB4B, RNU6-174P	2.16	0.43	-2.32	5.00E-05	0.034
ENSG00000060138	YBX3	5.61	9.91	0.82	5.00E-05	0.034
ENSG00000271417	SNORD114-26	6.31	2.79	-1.18	5.00E-05	0.034
ENSG00000129474, ENSG00000092036, ENSG00000265037, ENSG000000259132	AJUBA, HAUS4, MIR4707, RP11-298I3.5	12.72	7.55	-0.75	5.00E-05	0.034
ENSG00000221318, ENSG00000206127	AC135983.1, GOLGA80	12.61	0.21	-5.89	5.00E-05	0.034
ENSG00000008517, ENSG00000252561	IL32, RNU1-125P	1.66	2.92	0.82	5.00E-05	0.034
ENSG00000175061	LRRC75A-AS1	163.13	106.32	-0.62	5.00E-05	0.034
ENSG00000108691	CCL2	1.78	5.79	1.70	5.00E-05	0.034
ENSG00000267681, ENSG00000188554	CTD-3199J23.6, NBR1	4.33	36.72	3.08	5.00E-05	0.034
ENSG00000120071	KANSL1	0.43	0.95	1.12	5.00E-05	0.034
ENSG00000169136, ENSG00000265438	ATF5, MIR4751	3.09	13.02	2.08	5.00E-05	0.034
ENSG00000101187	SLCO4A1	3.67	8.32	1.18	5.00E-05	0.034
ENSG00000241158	ADAMTS9-AS1	1.40	3.90	1.47	5.00E-05	0.034
ENSG00000163638	ADAMTS9	5.66	14.55	1.36	5.00E-05	0.034
ENSG00000065060, ENSG00000252621	UHRF1BP1, Y_RNA	9.07	4.54	-1.00	5.00E-05	0.034
ENSG00000157593	SLC35B2	11.65	6.71	-0.80	5.00E-05	0.034
ENSG00000135245, ENSG00000165055, ENSG00000272601, ENSG00000273184	HILPDA, METTL2B, RP11-155G14.5, RP11-212P7.3	17.24	44.81	1.38	5.00E-05	0.034

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Ensembl Gene I.D.	Gene Symbol	Mean Normalized Counts (FDR = 0.05)				Q-value (%)
		Control	MDD	log2/sqrt	P-value (%)	
ENSG000000237268	RP13-492C18.2	2.62	0.67	-1.97	5.00E-05	0.034
ENSG00000199744, ENSG00000148303, ENSG000000252542	SNORD36A, RPL7A, SNORD36C	31.07	97.71	1.65	5.00E-05	0.034
ENSG00000204681, ENSG000000213886	GABBR1, UBD	3.41	1.55	-1.14	5.00E-05	0.034
ENSG000000147274, ENSG00000206979	RBMX, SNORD61	42.55	25.83	-0.72	5.00E-05	0.034