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ORIGINAL ARTICLE

Transcriptome profiling of human hippocampus dentate gyrus granule cells in mental illness

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This study is, to the best of our knowledge, the first application of whole transcriptome sequencing (RNA-seq) to cells isolated from postmortem human brain by laser capture microdissection. We investigated the transcriptome of dentate gyrus (DG) granule cells in postmortem human hippocampus in 79 subjects with mental illness (schizophrenia, bipolar disorder, major depression) and nonpsychiatric controls. We show that the choice of normalization approach for analysis of RNA-seq data had a strong effect on results; under our experimental conditions a nonstandard normalization method gave superior results. We found evidence of disrupted signaling by *miR-182* in mental illness. This was confirmed using a novel method of leveraging microRNA genetic variant information to indicate active targeting. In healthy subjects and those with bipolar disorder, carriers of a high- vs those with a low-expressing genotype of *miR-182* had different levels of *miR-182* target gene expression, indicating an active role of *miR-182* in shaping the DG transcriptome for those subject groups. By contrast, comparing the transcriptome between carriers of different genotypes among subjects with major depression and schizophrenia suggested a loss of DG *miR-182* signaling in these conditions.

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INTRODUCTION

Schizophrenia, bipolar disorder and major depression are common and severely disabling psychiatric conditions with a partially genetic background.[1–4] Family studies have shown co-aggregation of the major psychiatric disorders, and population-based studies have indicated shared genetic susceptibility loci.[5–9] Further evidence for common etiological factors comes from similarities in gene expression changes observed in different diseases, which have implicated deficits in neurotransmission and mitochondrial function, elevated immune response and inflammation, and downregulation of genes expressed in oligodendrocytes.[10–18] The goal of our study was to find common etiological mechanisms for these diseases through the identification of shared transcriptome changes.

One of the brain regions most consistently implicated in mental illness is the hippocampus, a brain region involved in memory, cognition, mood regulation and stress response.[19] In subjects with schizophrenia, bipolar disorder or major depression, abnormalities in hippocampus structure or function as well as a broad range of gene expression changes have been described.[12,13,20–28] The great majority of prior studies has been done in frontal cortex, but hippocampus has been repeatedly investigated as well.[29,30] However, interpreting changes in the hippocampal transcriptome is fraught with difficulty because of the different tasks performed by different hippocampal subregions. Consequently the areas CA1, CA3, and the dentate gyrus (DG) show large differences in gene expression; additional variability is introduced by functional differentiation along the long axis of the hippocampus.[31–34] The DG is of particular interest as it is one of only two brain regions where adult neurogenesis has been described.[35] A large body of literature has linked hippocampal neurogenesis with psychiatric illness, including affective disorders and schizophrenia.[36,37] We therefore chose to investigate the transcriptome of DG granule cells, isolating them from the surrounding tissue and harvesting them by laser capture microdissection (LCM).[38] We believe our study is the first to combine LCM with RNA-seq.

MATERIALS AND METHODS

Human subjects

Postmortem human brain tissue from 79 individuals was obtained from the Stanley Medical Research Institute (SMRI) Neuropathology Consortium, the UCLA Human Brain and Spinal Fluid Resource Center, and the University of Washington (UW) Neuropathology Core Brain bank. We investigated mid-hippocampus tissue from 79 subjects. Most ($n = 60$) subjects were from the SMRI Neuropathology Consortium, a well-described brain collection which has been extensively used in neuropsychiatric research.[39] The collection consists of four groups of 15 subjects each with schizophrenia, bipolar disorder, major depression and nonpsychiatric controls. Groups are matched by gender with nine males and six females per group, and by age, ranging from 25–68 years. Ten subjects, four males and five females ranging in age from 44–91 years old, were from the UCLA Human Brain and Spinal Fluid Resource Center. Of these, two carried a diagnosis of schizophrenia, one of bipolar disorder, two had suffered from major depression and five were nonpsychiatric controls. An additional nine nonpsychiatric controls were from the UW Neuropathology Core Brain bank. These subjects, five males and four females, ranged in age from 78–91 years.

The gender distribution (41–47% female) did not vary significantly by disease group (schizophrenia, bipolar disorder, major depression and nonpsychiatric controls). The mean age of our subjects did vary significantly by group ($P < 10^{-4}$), however, because our nonpsychiatric

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control subjects were on average older at death (64 ± 20 years) than members of the three disease groups, schizophrenia (47 ± 17 years), bipolar disorder (42 ± 16 years) and major depression (47 ± 17 years). We therefore evaluated the possible confounding influence of age by comparing DG transcriptomes between the seven youngest (age 19–44, mean 39 ± 5.7 years) and the six oldest (age 90–95, mean 92 ± 1.9 years) members of the healthy subject group. We identified four genes whose levels of expression appeared to be influenced by age (*MAGI2*, *RASGRF1*, *USP24* and *NUP107*). However, none of these genes were identified in any of our disease group comparisons, indicating that our results were not influenced by the age difference between psychiatric subjects and controls.

This study was approved by the Institutional Review Board of the University of Washington and conducted in accordance with ethics guidelines for the use of human subjects in research.

Laboratory methods

Fresh frozen 14 μm slide-mounted coronal cryostat sections from mid-hippocampus were stained and dehydrated using the Arcturus HistoGene LCM frozen section staining kit (Life Technologies, Grand Island, NY, USA), and following the manufacturer's instructions. From each subject, triplicate samples of about 2000 DG granule cells each were harvested by LCM, using an Arcturus AutoPix LCM system and CapSure Macro LCM caps (Molecular Devices, Sunnyvale, CA, USA). Triplicates were processed separately during cell harvest, RNA extraction and aRNA amplification to reduce experimental noise introduced during these stages of the experiment. Harvested cells were removed from the caps and RNA extracted using PicoPure RNA isolation kits (Life Technologies). RNA was then linearly amplified over two rounds of aRNA amplification, using MessageAmp II aRNA amplification kits (Life Technologies), and following the manufacturer's protocol. The quality and concentration of aRNA was checked by spectrophotometry, and only samples with an A260/280 ratio >1.9 were used. Equimolar amounts of triplicate aRNA samples for each of the 79 subjects were then pooled for the preparation of sequencing libraries. To evaluate different normalization/scaling methods, a separate test data set was prepared. In this data set, duplicate aRNA samples (denoted A and B) from four randomly chosen subjects (T1–T4) were used to construct a set of eight libraries.

Sequencing libraries were prepared using Total RNA Sequencing Kit (Life Technologies), following the directions for Whole Transcriptome Libraries, and analyzed with an Applied Biosystems SOLiD 4 high-throughput sequencer with an average singe-end read length of 50 base pairs (bp) (Life Technologies).

The genetic polymorphism rs76481776 of *miR-182* was genotyped using a StepOnePlus Real-Time PCR System and a TaqMan Custom SNP Genotyping Assay (Life Technologies). A amount of 50 ng genomic DNA was amplified in the presence of gene-specific primers and allele-specific fluorescent probes following the manufacturer's instructions. Genotypes were called using TaqMan Genotyper software. For quality control 10% of the samples were genotyped in duplicate, and the genotype distribution was tested for deviation from Hardy–Weinberg equilibrium, using a χ^2 test.

Data analysis

Reads were mapped and counted using the Applied Biosystems software BioScope 1.2.1. Transcripts were mapped to genome build GRCh37/hg19 (February 2009 assembly), using the UCSC RefGene annotations and the BioScope default seed-and-extend approach for mapping. Reads were mapped to the whole genome file, supplemented by a transcript annotation file allowing reads to align across known splice junctions with no gap penalty. Repeat and ambiguously mapped sequences were then removed from the counts files using a UCSC RepeatMasker file. We then used the BEDTools bamToBed program to generate files of read locations.[40] We only counted reads mapping to the opposite strand. As in RNA-seq, even a gene with a single count among multiple subjects will be listed as 'expressed' in the sequencer output, very low-expressing genes need to be excluded by a threshold criterion to limit experimental

Table 1. Clustering of samples according to different normalization/scaling strategies

Normalization method	*Use of gene length data*	*Scaling*	*Fraction*	*Sample*								
				T1		*T2*		*T3*		*T4*		
				A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*Number of falsely grouped samples*
None	Raw data			2	2	4	3	3	3	2	1	2
1	Exon or transcript length not considered			4	4	2	1	2	1	4	3	3
2	Reads multiplied by exon length	R	N	3	3	2	4	2	4	1	1	2
3	Reads divided by exon length	R	N	4	4	4	3	1	3	2	2	2
4	Reads multiplied by transcript length	R	N	2	2	2	3	3	3	1	4	2
5	Reads divided by transcript length	R	N	3	2	1	4	1	4	1	1	3
6	Reads multiplied by exon length	S	N	3	3	4	2	4	2	1	1	2
7	Reads divided by exon length	S	N	3	3	3	2	4	1	4	4	2
8	Reads multiplied by transcript length	S	N	1	1	1	4	4	4	2	3	2
9	*Reads divided by transcript length*	*S*	*N*	*4*	*4*	*1*	*1*	*2*	*1*	*3*	*3*	*1*
10	Reads multiplied by exon length	R	Y	1	1	2	3	2	3	4	4	2
11	Reads divided by exon length	R	Y	3	3	3	2	4	2	1	1	2
12	Reads multiplied by transcript length	R	Y	4	4	4	2	1	2	3	3	2
13	Reads divided by transcript length	R	Y	4	4	2	1	3	1	2	2	2
14	Reads multiplied by exon length	S	Y	3	3	1	2	1	4	3	3	4
15	Reads divided by exon length	S	Y	2	2	2	4	3	1	3	3	2
16	**Reads multiplied by transcript length**	**S**	**Y**	**3**	**3**	**4**	**4**	**1**	**1**	**2**	**2**	**0**
17	*Reads divided by transcript length*	*S*	*Y*	*4*	*4*	*3*	*3*	*2*	*3*	*1*	*1*	*1*

Shown are cluster memberships for technical replicates (A and B) of four randomly chosen samples (T1–4). The principles guiding the different normalization strategies with respect to transcript or exon length are shown in column 2. Column 3 delineates the choice of two different scaling methods: R—counts are divided by the total number of reads per sample; S—counts are scaled to the total sum of gene × length products or quotients per sample. Column 4 indicates whether individual transcript reads are divided by the total number of mappable reads before entering the equation (N—no; Y—yes), that is, whether reads per transcript are considered as a fraction or all reads or not. A full description is given in the Methods section. The labels 1, 2, 3 and 4 define the four clusters; samples that belong to the same cluster receive the same label. Method 3 is analogous to the RPKM method. Only method 16 (bold) leads to the correct clustering. Methods 9 and 17 (italics) give the same clustering results—only the numbering of the clusters is changed—and perform slightly worse than method 16, with one falsely grouped sample (T3B).

noise.[41,42] We thus only considered genes to be expressing at analyzable levels if their raw counts were greater than zero in at least 95% of subjects, that is, zero in no more than three of our 79 samples. For our test data sets which were used for the evaluation of different normalization strategies, this meant that all genes without mappable reads in any one of the eight samples were excluded (since one out of eight would have amounted to 12.5% zero reads).

The average number of total mapped reads per subject was 16 357 257. The average fraction of uniquely mapped reads was 45%. For multiple transcripts at a given genomic location, for example, owing to the presence of splice variants, only one transcript with the highest number of counts was included in the analyses. A total of 15 761 of the resulting 22 075 transcripts failed our threshold criterion of expression above background; the remaining 6314 transcripts/genes were analyzed with regard to disease-specific expression profiles. Our test data set, used for the evaluation of different normalization strategies, contained 9858 transcripts. The reason that our test data set contained a third more transcripts than the analysis data set lies with our exclusion of genes expressing at background, which led to a higher number of genes being dropped from the analysis data set.

For the evaluation of different normalization approaches, we compared a panel of 17 different methods plus raw (non-normalized) data. Normalization methods differed by their use of exon vs transcript length data and different scaling approaches (Table 1; also see Supplementary Methods for a comprehensive mathematical description). Each of these 18 approaches was applied to our test data set consisting of technical replicates from four subjects (T1–T4). We employed k-means clustering in an attempt to recover the four natural clusters in which technical replicates are paired, and evaluated methods by how well they recovered the natural clusters.

To account for the fact that genes act cooperatively in biological systems, we developed a new analysis approach for comparison of transcriptome profiles which is based on the identification of genes which are, given the presence of all other genes, significantly involved in shaping a specific gene expression profile. This regression-based analysis approach, identified by the acronym SIcall (for 'significantly involved calls') is described in detail in the Supplementary Methods. To infer microRNA (miRNA) involvement from groups of significantly involved genes, we used TargetScan (Release 6.3, June 2012). Genotype by target gene expression interactions were modeled using two-way analysis of variance (ANOVA) models (see Supplementary Methods for details).

RESULTS

Superior performance of nonstandard normalization methods in postmortem human brain

LCM combined with aRNA amplification from postmortem human brain is a powerful technique to obtain cell population specific transcriptome data, but poses technical challenges. RNA from postmortem human brain is subject to degradation owing to agonal factors and a postmortem interval between death and the preservation of tissue. This problem is compounded by LCM, during which some amount of RNA degradation inevitably occurs even with stringent RNAse-free technique.[43] In addition, aRNA production leads to shortening of transcriptomes over successive cycles of amplification.[44–46]

In the most widely used normalization strategy for RNA-seq experiments, the RPKM method (reads per kilobase of exon model per million mapped reads), dividing raw counts by exon length reduces the bias that is introduced by the fact that longer genes accumulate more counts.[47] In our sample of 96 subjects, average mapped reads (raw counts) were more strongly proportional to total transcript length ($r = 0.427$, $P < 10^{-4}$) than to cumulative exon length, however ($r = 0.080$, $P < 10^{-4}$) (see also the Supplementary Figure).

In principle, the shortening of measurable transcripts occurring as a result of partial mRNA degradation and aRNA amplification under our experimental condition disproportionally affects shorter genes. For example, a loss of 500 bp will remove 50% of the signal from a 1-kb transcript, but only 25% of the signal of a 2-kb mRNA. As a result, in our experiment shorter genes had 'noisier' levels of expression, as indicated by higher coefficients of variation of the raw mapped counts. This inversely proportional relationship was stronger for total transcript length ($r = -0.104$, $P < 10^{-4}$) than for exon length ($r = -0.063$, $P < 10^{-4}$).

To answer the question which normalization method would perform best under our experimental conditions, we designed and tested 17 different methods, plus no normalization. K-means clustering was used to compare the quality of different approaches, based on the assumption that the best performing method(s) would appropriately cluster technical replicates together and samples from the four different subjects as distinct. Only one method (#16) led to the correct clustering; two other methods produced results that were identical to each other with one subject placed in the wrong cluster (#9 and #17, Table 1). The top-performing method filters out a portion of the experimental noise by introducing a stronger bias against noisier, shorter transcripts, whereas the two runners-up reduce the inherent sequencing bias against short transcripts in a way that is similar to the RPKM method. As the top-performing method, noise reduction scaling (#16) became the basis for our subsequent analyses. Length scaling (# 9) was used to compare the effects of different normalization strategies, and to confirm results.

Identification of transcriptome differences between subject groups

We made the following seven comparisons (1) all mental illness ($n = 50$) vs nonpsychiatric controls ($n = 29$), (2) schizophrenia ($n = 17$) vs nonpsychiatric controls ($n = 29$), (3) bipolar disorder ($n = 16$) vs nonpsychiatric controls ($n = 29$), (4) major depression ($n = 17$) vs nonpsychiatric controls ($n = 29$), (5) schizophrenia ($n = 17$) vs bipolar disorder ($n = 16$), (6) schizophrenia ($n = 17$) vs major depression ($n = 17$), (7) bipolar disorder ($n = 16$) vs major depression ($n = 17$). Cumulatively these comparisons identified 141 genes as likely to be involved in shaping DG expression profiles in mental illness (Supplementary Table 1). In contrast the 'fold change' output of traditional regression methods, the weights given by SIcall analysis are a rough measure of the likelihood of a gene contributing to overall DG gene expression changes in mental illness, given the presence of all other genes represented in the transcriptome.

Influence of the normalization and analysis method on gene identification

Using an alternative normalization method, length scaling instead of noise reduction scaling led to the identification of 162 genes. Only 64 of these genes, in particular the most heavily weighted ones, were identified using both scaling methods. Hence, identification of the majority of genes was strongly dependent on the choice of normalization method. For the 64 genes identified in both versions of the scaled data, there was fairly good overlap in the pattern of weights generated by our different comparisons (Supplementary Table 2).

The dependence of gene identification on normalization method was independent of the type of analysis method used. Using univariate logistic regression models led to the identification of 65 genes as differentially expressed (any of seven group comparisons, $P < 0.01$) using length scaled data, and 80 genes as differentially expressed (any of seven group comparisons, $P < 0.01$) using noise reduction scaling, with an overlap of 26 genes between the two normalization methods (data not shown). Hence, for either analysis method (SIcall vs a more traditional approach) only about a third of genes were reproducibly identified across different normalization methods.

Deducing miRNA involvement from transcriptome data

We next investigated the possibility that our observed DG transcriptome changes in mental illness could be the result of dysregulated miRNA signaling. The deduction of miRNA involvement from mRNA gene expression profiles relies on computational approaches that match miRNAs to target genes by searching the 3′UTR of potential miRNA target genes for 6–8 bp miRNA binding sites. Yet only a subset of miRNA binding sites and target genes identified by purely computational approaches is biologically relevant.

To address this problem we applied a two-step approach in which we used our most heavily weighted genes (Set 1, weights of 4 or greater, $n = 21$) to discover possible miRNA involvement, using TargetScan. Genes with a total comparison weight 1–3 were assigned to Set 2 ($n = 117$) (Supplementary Table 1). We hypothesized that if DG transcriptome changes in psychiatric conditions result at least to some extent from dysregulation of signaling by a miRNA, both heavily weighted (Set 1) and more lightly weighted genes (Set 2) should have overrepresentation of target genes for this particular miRNA compared with the remainder of transcripts expressing above background but not identified by SIcall ('non-called genes', NC, $n = 6055$). We further hypothesized that targeting by this miRNA would be strongest in Set 1 genes, followed by Set 2 genes, followed by NC genes.

Twenty miRNAs or miRNA families targeted at least 25% (⩾5) of Set 1 genes (Supplementary Table 3). Among these, two followed the hypothesized pattern of a drop in targeting rates from Set 1 over Set 2 to NC genes, with statistically significant differences in the number of targeted genes between Set 2 and NC genes: *miR-182* and the miR-30abcdef/30abe-5p/384-5p family. After Bonferroni correction for the number of χ^2 tests performed, only *miR-182* remained statistically significant (Table 2).

A higher proportion of *miR-182* target genes among genes identified by SIcall compared with NC genes was also observed if length scaling was used for normalization. A total of 27 of 158 genes identified by SIcall (any weight) in our method 9 normalized data set were *miR-182* targets, compared with 636 targets among the corresponding 6035 NC genes ($\chi^2 = 6.9$, $P = 0.009$).

Table 2. Targeting of SIcall and control gene sets by microRNA (miRNA)

miRNA/miRNA family	*% Of genes targeted*			*Set 2 vs NC*	
	Set 1	*Set 2*	*NC*	X^2	P
miR-29abcd	40	11	9	0.71	0.399
miR-518a-5p/520d-5p/524-5p	35	14	18	1.21	0.272
miR-182	30	22	10	16.81	0.00004
miR-3148	30	12	10	0.29	0.593
miR-548ah/3609	30	9	8	0.15	0.701
miR-548c-3p	25	14	18	1.65	0.199
miR-677/4276	30	5	4	0.30	0.585
miR-1326/4766-5p	25	5	6	0.14	0.705
miR-15abc/16/16abc/195/322/424/497/1907	25	9	11	0.39	0.534
miR-181abcd/4262	25	10	12	0.26	0.609
miR-30abcdef/30abe-5p/384-5p	25	21	13	5.90	0.015
miR-3613-3p	25	9	12	0.72	0.395
miR-3714	25	16	11	2.71	0.100
miR-4282	25	13	13	0.03	0.856
miR-4698	30	9	11	0.34	0.559
miR-4796-3p	25	6	6	0.02	0.903
miR-513a-5p	25	8	8	0.02	0.890
miR-607	25	18	13	3.09	0.079
miR-9/9ab	25	13	11	0.33	0.566
miR-96/507/1271	25	10	10	0.03	0.872

Listed are all miRNAs/miRNA families targeting at least five (25%) of Set 1 genes. The % of target genes among Set 1 genes ($n = 20$), Set 2 genes ($n = 117$) and non-called genes (NC, transcripts expressing above background, but not identified by significantly involved calls (SIcall, $n = 6055$)) are given. For each miRNA, the numbers of target genes vs nontarget genes in Set 2 vs NC genes were compared using χ^2 tests, with *P*-values shown in the rightmost column.

Validation of *miR-182* involvement in shaping DG granule cell transcriptomes

Saus *et al.*[48] have shown that a *C* to *T* substitution in the single-nucleotide polymorphism rs76481776 leads to overexpression of *miR-182* in *T*- vs *C*-allele carriers and causes a significant reduction in target gene expression. The minor allele frequency of rs76481776 in our subjects was 8.9%, that is, 13 of our 79 subjects were *T*-allele carriers, one of them a *T/T* homozygote; the remaining 66 individuals had the *C/C* genotype. Our minor allele (*T*) frequency of 8.9% was in good agreement with the previously reported 7.5% in Spanish subjects.[48] Genotypes were in Hardy–-Weinberg equilibrium (not shown).

We hypothesized that whenever *miR-182* was active in shaping DG gene expression profiles, we would be able to observe a statistically significant difference in target gene expression between T-allele carriers (*C/T* or *T/T* genotype) and those with the *C/C* genotype. On the other hand, no difference in *miR-182* target gene expression between rs76481776 *T*-allele carriers vs noncarriers would indicate that *miR-182* is not actively involved in regulating the transcriptome. To use the functional variant rs76481776 as a detector of miRNA-182 action on the transcriptome, we compared the expression levels of *miR-182* target genes between carriers and noncarriers of the uncommon *T*-variant in each of our three disease groups (schizophrenia, bipolar disorder, major depression) and in nonpsychiatric controls.

The differences in the mean *miR-182* target expression levels between carriers and noncarriers of the rs76481776 *T*-allele varied as a function of the psychiatric diagnosis ($F = 13.10$, $P < 10^{-4}$). We observed significant differences in mean *miR-182* target expression levels between carriers and noncarriers of the *T*-allele in nonpsychiatric controls ($t = 4.77$, $P < 10^{-4}$) and in subjects with bipolar disorder ($t = -3.48$, $P < 10^{-4}$). By contrast, target gene expression levels did not differ significantly by genotype group in individuals with schizophrenia ($t = -1.61$, $P = 0.108$) or major depression ($t = 0.88$, $P = 0.380$). Hence, although *miR-182* targeting is active in DG granule cells of control subjects and individuals with bipolar disorder, it appears to be lost in subjects with schizophrenia and major depression. Using our alternative normalization method (length scaled data) we could confirm loss of *miR-182* signaling in subjects with depression, but not in schizophrenia (data not shown).

DISCUSSION

This study is, to the best of our knowledge, the first application of RNA-seq to cells isolated from postmortem human brain by LCM. Our whole transcriptome analysis approach, SIcall, will be a useful addition to the tool chest of other currently available analysis methods. To our knowledge, our study is also the first to use genetic methods for validation of miRNA gene targeting in global transcriptomes.

Gene expression studies in humans are made difficult by the marked heterogeneity of subjects, which strongly reduces statistical power.[49] We therefore tried to maximize our power to detect differences between groups by collecting a larger subject group, using samples from three different brain banks. Nonetheless, variability of subject and sample characteristics creates important confounders in transcriptome comparisons. Previous studies have indicated that RNA quality, brain pH, postmortem

interval, subject gender, ethnicity, age, disease duration, drug treatment history, suicide status, alcohol and substance abuse comorbidity can affect results.[13] Among these, RNA integrity and the factors directly affecting it such as postmortem interval and brain pH have by far the strongest impact.[50,51] Analysis of postmortem human brain has shown that a longer postmortem interval and lower tissue pH lead to decreased RNA integrity, which introduces noise into gene expression data.[52–54] This is further exacerbated by a previously demonstrated 30% drop in RNA integrity during LCM.[43] Other covariates may create false positive reports of differential gene expression or mitigate true gene expression differences. For example, a previous study of SMRI samples has shown that higher cumulative lifetime antipsychotic dose probably normalizes some of the inherent molecular changes of schizophrenia.[55] Additional difficulty is created by the fact that not all relevant subject information might be known. For example we did not have access to family psychiatric history or lifetime exposure to psychotropic drugs for subjects from the UCLA and UW brain banks. We investigated the influence of subject age, our potentially most relevant confounder, on transcriptome differences, and found that it did not affect our results. Given the large number of potentially confounding subject variables, however, we cannot exclude the possibility that other confounders might have influenced our observed gene expression patterns.

The statistical analysis of transcriptome data traditionally relies on separate comparison of expression levels for each gene between case and control conditions, resulting in a report of fold changes for each gene. To explore the cooperative action of groups of genes, we used an alternative regression-based analysis approach (SIcall) looking at the simultaneous actions of up to five genes. Although models with higher numbers of participating genes are possible, their computational cost is prohibitive. For each model, our algorithm generates a large number of logistical regressions representing the many ways in which small groups of genes can cooperatively characterize transcriptome differences between two groups. Similar approaches have previously been used in the analysis of gene expression data.[56] For each comparison we set the threshold of the probability at which a gene would be considered involved in shaping gene expression profiles to 0.05. In other words, if a gene had at least 5% probability of being featured in one of five sets of logistic regression models allowing for the simultaneous action of either 1, 2, 3, 4 or 5 genes at a time, it was listed as significantly involved and entered our subsequent analysis steps. It should be noted that this 5% represents an empirically chosen probability threshold which does not correspond to statistical significance. Genes were weighted by the number of times they were called per comparison (up to five), and the total weights across all seven comparisons (up to a theoretical maximum of 35). It should be noted that total gene weights are not quantitative in the way gene expression changes are, but they rather represent rough measures of the likelihood of the involvement of a given gene. Only the results of the simplest SIcall models, such as those which investigate one gene at a time, roughly correspond to the traditional idea of gene-by-gene differential expression.

Including individual disease vs disease comparisons (e.g. schizophrenia vs major depression) in our analysis was based on the hypothesis that any gene significantly involved in shaping a disease-specific transcriptome might also reveal itself in comparison of this disease with any other psychiatric condition. For example, the gene *C9orf102* is heavily weighted in both the bipolar disorder vs control, and the bipolar disorder vs depression comparisons. We can hypothesize from this that *C9orf102* expression might potentially be useful as a biomarker differentiating bipolar disorder from major depression, warranting further experimental exploration and confirmation. The genes *OPTN*, *FAM124A*, *OXSR1*, *RLF* and *TLL1* are heavily weighted in the comparison of schizophrenia against major depression, but are not called in any other comparison. Our analysis does not reveal which of these genes might be involved in schizophrenia, which in depression, or which in both, the latter as a result of opposing gene expression changes in the two conditions. Nonetheless, the fact that they are called by our analysis indicates that investigating them further might yield insights into broad processes which may be dysregulated in major depression or schizophrenia. Our inclusion of an 'all disease' vs nonpsychiatric control comparison was motivated by the hypothesis that major psychiatric conditions might share subtle transcriptome changes that are detectable only if larger groups are compared. However, contrary to this expectation, there were relatively few genes that had weights >1 and were called only in the all disease vs control, but not in any other comparison.

Prior gene expression studies in postmortem human brain have successfully implicated broad systems dysfunction in mental illness.[10–15] Most of these studies have used microarray technology, but RNA-seq has been employed in more recent work.[16–18] The vast majority of prior studies investigated tissue blocks as opposed to near-homogeneous cell populations isolated by LCM. One previous study exists in which DG gene expression profiles were compared in subjects with schizophrenia, bipolar disorder, major depression and nonpsychiatric controls, using LCM and microarrays.[57] The authors found decreased expression of genes related to protein turnover, energy metabolism and neuronal functions in subjects with schizophrenia compared with controls. No significant transcriptome changes were observed in subjects with major depression or bipolar disorder.

Although prior gene expression studies have been consistent in reporting systems-level dysfunction in the brains of subjects with mental illness, for example, inflammation, observations of differential expression for individual genes have been far less reproducible. In a meta-analysis of 12 genome-wide expression studies in postmortem brain of subjects with bipolar disorder compared with controls, Elashoff *et al.*[14] have shown that the likelihood of a gene reported as differentially expressed in one study having a repeat finding in one of 11 other studies was only 9%. This lack of robustness in gene expression findings has previously been attributed to interacting factors such as tissue pH and subject age or gender.[13] Our findings indicate that the choice of suboptimal normalization methods may be an additional contributing factor. Under our experimental conditions the generally accepted standard of transcriptome normalization, the RPKM method, showed inferior performance compared with the other approaches.

We hypothesized that our observed gene expression changes might have occurred as the result of an overarching dysregulation of gene expression in mental illness. We chose to look at posttranscriptional regulation by miRNAs because of strong evidence of their involvement in DG neurogenesis and major psychiatric disorders. The miRNAs are a class of small noncoding RNAs which inhibit expression of groups of target genes through degradation or inhibition of their mRNAs. Over half of miRNAs are highly or exclusively expressed in brain, where they participate in neurogenesis and neuronal plasticity.[58–65] Changes in miRNA expression in postmortem human brain have previously been shown in schizophrenia,[66–74] bipolar disorder[72,73] and depression.[75]

Contrasting with our approach, prior studies have relied on the direct profiling of miRNA expression using a preselected panel of miRNAs. Accurate direct profiling of miRNAs in postmortem human brain could be compromised, however, by potentially limited miRNA stability in neuronal cells. Although studies in embryonic cell lines and peripheral organs such as liver and heart have shown that miRNAs can be highly stable molecules with half-lives of up to several days,[76–78] comparing miRNA decay in neurons with that in non-neuronal cells, Krol *et al.*[79] found miRNA decay in neurons to be activity-dependent, and occurring much

faster than in non-neuronal cells. Their observation of miRNA half-lives of less than 1 h agrees with other findings in human primary neuronal cells and short postmortem interval human neocortex, where Sethi and Lukiw[80] reported miRNA half-lives ranging from 1 h to about 3.5 h. The latter would mean that during a postmortem interval of 24 h, which is the case for many human subjects in publicly available brain collections, more than 99% of miRNA molecules have degraded.[50] Our ability to directly detect changes in miRNA expression in our subjects was further compromised by our use of aRNA amplification. Although miRNAs are translated from polyadenylated transcripts, the poly-A tail is lost in mature miRNAs. Hence the bulk of mature miRNAs was lost during aRNA amplification before the preparation of sequencing libraries. Possibly as a result of that, we were not able to detect expression of *mir-182* or any member of the miR-30 family in our subjects.

We believe our study is the first to show evidence of disrupted *miR-182* signaling in schizophrenia and major depression. Members of the miR-30 family, however, have been repeatedly shown to have decreased expression in schizophrenia.[81] It should be noted that only a minority of genes identified in our RNA-seq analysis were actually *miR-182* targets. Hence, it is clear that we were able to discover only one of possibly a multitude of regulatory mechanisms accountable for shaping DG transcriptome changes in mental illness.

miR-182 is part of a cluster of three miRNAs, *miR-96*, *miR-182* and *miR-183*, which are colocated within a 4 kb genomic segment located at 7q32.2.[82,83] *miR-182* is involved in a broad range of biological processes including regulation of the immune response, DNA repair, cell proliferation and differentiation, and regeneration of peripheral nerves after injury.[84–89] *miR-182* is the highest expressing miRNA in the pineal gland, where it accounts for 28% of the miRNA population. There, *miR-182* has a rhythmic pattern of gene expression involving approximately two-fold changes between the highest levels of expression between 6 am and 12 pm, and the lowest levels during the night.[90] In keeping with this, Saus *et al*.[48] found an association between the rs76481776 polymorphism of the *miR-182* gene and patterns of insomnia in patients with major depression. Disruption of sleep and circadian rhythmicity are cardinal features of both schizophrenia and major depression, which aligns with our findings of a possible loss of *miR-182* signaling in these conditions.[91–93]

CONCLUSIONS

Whole transcriptome analysis by RNA-seq in LCM-isolated DG granule cells of postmortem hippocampus in subjects with mental illness and controls showed evidence of disrupted *miR-182* signaling in subjects with major depression and schizophrenia. We validated this finding by showing how the impact of a functional *miR-182* single-nucleotide polymorphism on target gene expression was lost in subjects with schizophrenia and major depression. Our paper is the first study in which LCM is combined with RNA-seq in postmortem human brain. Under these challenging experimental conditions, a noise reduction scaling normalization method outperformed normalization by exon or transcript length. We also demonstrate the feasibility of a novel, regression-based method for RNA-seq analysis (SIcall) which allows for the investigation of cooperative action among small sets of genes and is a useful complement to existing approaches.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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