

ORIGINAL ARTICLE

Patterns of gene expression in the limbic system of suicides with and without major depression

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The limbic system has consistently been associated with the control of emotions and with mood disorders. The goal of this study was to identify new molecular targets associated with suicide and with major depression using oligonucleotide microarrays in the limbic system (amygdala, hippocampus, anterior cingulate gyrus (BA24) and posterior cingulate gyrus (BA29)). A total of 39 subjects were included in this study. They were all male subjects and comprised 26 suicides (depressed suicides = 18, non depressed suicides = 8) and 13 matched controls. Brain gene expression analysis was carried out on human brain samples using the Affymetrix HG U133 chip set. Differential expression in each of the limbic regions showed group-specific patterns of expression, supporting particular neurobiological mechanisms implicated in suicide and depression. Confirmation of genes selected based on their significance and the interest of their function with reverse transcriptase-polymerase chain reaction showed consistently correlated signals with the results obtained in the microarray analysis. Gene ontology analysis with differentially expressed genes revealed an over-representation of transcription and metabolism-related genes in the hippocampus and amygdala, whereas differentially expressed genes in BA24 and BA29 were more generally related to RNA-binding, regulation of enzymatic activity and protein metabolism. Limbic expression patterns were most extensively altered in the hippocampus, where processes related to major depression were associated with altered expression of factors involved with transcription and cellular metabolism. Additionally, our results confirm previous evidence pointing to global alteration of gabaergic neurotransmission in suicide and major depression, offering new avenues in the study and possibly treatment of such complex disorders. Overall, these data suggest that specific patterns of expression in the limbic system contribute to the etiology of depression and suicidal behaviors and highlight the role of the hippocampus in major depression.

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Introduction

Suicide is an important public health problem that has a strong association with psychopathology and particularly with mood disorders.^{1,2} There is a large body of evidence suggesting that neurobiological factors play a role increasing predisposition to suicide.^{3,4} In spite of some overlap, this neurobiological predisposition seems to be, to a considerable extent, different from that mediating risk to major depression or other psychopathological processes commonly present in suicide completers.⁵

Neuroimaging studies have produced a substantial body of knowledge about alterations of the limbic system in mood disorders. In the amygdala, alterations in cerebral blood flow and metabolism,⁶ asymmetry of amygdalar volumes,⁷ as well as smaller^{8–10} and larger volumes^{11–14} have been observed in depressed subjects when compared with normal controls. In the hippocampus, volumetric analysis studies have also revealed reduced volumes in subjects suffering from major depression in some,^{7,11,14–20} but not all studies^{10,21–23} comparing depressed patients versus controls. Alterations in the cingulate cortex have also been observed by many authors. Among these, a smaller anterior cingulate (or Brodmann Area 24: BA24) was observed in depressed patients when compared to controls^{9,24,25} and altered activity has also been reported.^{26–28} Brodmann area 29 (BA29) or posterior cingulate, has been associated mainly to response to antidepressant treatment with changes in metabolic response associated with the different treatments.^{28–31} The observed changes in these limbic

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regions may modulate the risk of suicidal behavior through their influence on depression or stress response. In particular, the involvement of the hippocampus in depression and suicidal behavior has been inferred from studies revealing morphological changes of this structure in response to stress hormones, although these changes may often be reversible markers of an ongoing stress or depression-related process.³²

Also, postmortem examinations have produced a large body of evidence supporting the implication of limbic areas in suicide. Molecular studies in postmortem hippocampi of suicides have pointed to altered levels of 5-HT_{2A} receptor,³³ cyclic AMP (cAMP) response element-binding,³⁴ extracellular signal-regulated kinase1/2 mitogen-activated protein (MAP) kinases,^{35,36} protein kinase C isozymes.³⁶ Additionally, in a recent study, Rosel *et al.*³⁷ observed altered levels of 5-HT_{2A} and 5-HT₄ receptors and their respective intracellular signaling systems IP₃ and cAMP in hippocampi of suicides when compared to controls. A number of recent investigations have highlighted the potential for identification of genetic predisposing factors for depression³⁸ and suicidal behavior,³⁹ including WFS1^{40–42} and p75NTR,⁴³ among others. However, these studies have focused on expression levels of one or several genes at a time and compared suicides with or without psychopathology to controls, not providing an idea of the overall changes taking place in relation to suicide versus the ones related to major depression. Although many of these findings may be biologically relevant, they are difficult to confirm in the absence of larger studies seeking to replicate these findings in similar clinical samples with comprehensive coverage of the variants at a particular candidate locus. It is therefore important that genome-wide analyses of gene expression accompany these focused efforts to better understand the relative significance of previously reported findings and direct attention toward particular biochemical pathways and processes.

A growing number of microarray-based investigations have been conducted in recent years; however, relatively few have examined complex behavioral phenotypes, especially in humans. There is good evidence for differential gene expression underlying complex phenotypes, as in the case of avoidance learning in rodents.⁴⁴ In this study, using oligonucleotide microarrays for high-throughput analysis of mRNA levels in the limbic system (hippocampus, amygdala, BA24 and BA29), we compared the expression patterns of male subjects suicides with and without major depression and psychiatrically normal controls.

Materials and methods

Subjects

Subjects were all males of French-Canadian origin, a homogeneous population with a well-known founder effect.⁴⁵ Both cases and controls were age and post-

mortem interval (PMI) matched. All subjects died suddenly without a prolonged agonal state or protracted medical illness. Brain samples were obtained from the Quebec Suicide Brain Bank and were collected with PMI of less than 36 h at autopsy. Amygdala, hippocampus, anterior cingulate gyrus (BA24) and posterior cingulate gyrus (BA29) were sampled at 4°C and snap-frozen in liquid nitrogen before storage at –80°C. This study was approved by our local IRB and informed consent was obtained from next of kin.

All subjects were psychiatrically characterized by psychological autopsies, which are validated methods to reconstruct psychiatric history by means of extensive proxy-based interviews, as outlined elsewhere.⁴⁶ The sample consisted of subjects who committed suicide during an episode of major depression (SMD; *n* = 18); suicide victims (S; *n* = 8) with no lifetime history of major depression; and matched controls (C; *n* = 13) with no history of suicidal behavior or a major psychiatric diagnosis.

Microarray analysis

Sample processing, RNA extraction, RNA quality control and gene expression by microarray were performed at Gene Logic Inc. (Gaithersburg, MD, USA, www.genelogic.com). All microarray data and clinical information were embedded into Gene Logic's Genesis 2.0 software as a component of its Bioexpress system. RNA samples used in the current study had a minimum A260/A280 ratio of 1.9. The samples were further checked for evidence of degradation and integrity. Samples had a minimum 28S/18S ratio of >1.6 (2100-Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). We used the Human Genome U133 Set, which consists of two GeneChip arrays with ~45 000 probe sets representing >39 000 transcripts derived from ~33 000 well-substantiated human genes (available at <http://www.affymetrix.com>).

GeneChip analysis was performed in Genesis 2.0 (Gene Logic Inc.) and with Microarray Analysis Suite (MAS) 5.0, Data Mining Tool 2.0, and Microarray Database software (available at <http://www.affymetrix.com>). All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 100.

Several microarray RNA integrity indicators were used in this study to filter samples for quality for final analysis. Principal Component Analysis (PCA) was used to quickly identify outlier arrays. Microarray quality control parameters included the following: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, and consistent β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'/3' signal ratios. Outlier arrays subjects were excluded on a region basis without any subject being excluded from all the regions. Similar number of subjects were included in the final analysis across the three regions (amygdala: C = 8, S = 6, SMD = 14; hippocampus: C = 6, S = 6, SMD = 10; BA24: C = 7, S = 5, SMD = 9; BA29: C = 8, S = 7, SMD = 10).

Data analysis

For a gene to be included in the final analysis, it had to be 'present' (according to MAS 5.0) in at least 75% of the subjects in at least one of the three groups to reduce the chances of false-positive results and to exclude bad probe sets. Expression data was analyzed using Genesis 2.0 (GeneLogic Inc., Gaithersburg, MD, USA) and AVADIS software (Strand Genomics, Redwood City, CA, USA). Gene expression values were floored to 1 and then \log_2 transformed.

Analysis of variance (ANOVA)s were performed for each gene to identify statistically significant gene expression changes. To identify differences between the SMD, suicides (S), and controls (C), statistically significant genes were subjected to a *post hoc t*-test for the contrasts SMD versus C, S versus C, and SMD versus S. Two criteria were used in all to determine whether a gene was differentially expressed. First, a gene had to have an ANOVA *P*-value of less than or equal to 0.01. Second, for a given contrast a gene had to have a fold change/*P*-value combination of at least 1.3-fold change in both direction and $P \leq 0.01$.

Cluster analysis was performed using average-linkage hierarchical cluster analysis with a correlation metric. Both expression patterns in individuals and genes were clustered. PCA was performed based on the initial gene sets and on the selected genes (according to our significance criteria). PCA based on the initial gene set did not discriminate the groups. PCA based on the selected genes showed discrimination of the three groups.

Gene ontology analysis was conducted for differentially expressed genes using the enrichment algorithm integrated in the Database for Annotation, Visualization and Integrated Discovery (DAVID 2.0). DAVID is a web-based application that allows users to access relational databases for functional annotations.⁴⁷

Real-time PCR

Total RNA was re-extracted from 10–20 mg of frozen tissue in the four areas examined using Trizol (Invitrogen Corp, Carlsbad, CA, USA). These samples were independent from those used in the microarray assays, and were obtained from adjacent tissue dissections. Quality of the RNA was established using OD measurements and evaluation on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), with samples below an RNA integrity number of 5 excluded from further analysis. Synthesis of complementary DNA (cDNA) from 1 μ g of total RNA was carried out by oligo(dT)-priming using SuperScript II reverse transcriptase (Invitrogen Corp). Real-time analysis of expression results for genes of interest (spermidine/spermine *N*-1-acetyltransferase gene (SSAT)1, SSAT2, OATL1, SYT4, ADCY8, APLP2 and BACE1) was carried out using TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) with 5 ng of cDNA template in a 15 μ l reaction volume. The polymerase chain reaction (PCR) reactions were run on an ABI 7900HT Real-Time PCR

system (Applied Biosystems) according to the manufacturer specified conditions (ABI TaqMan gene expression assays protocol, Rev E). Fold changes between groups were evaluated using relative quantitation ($\Delta\Delta C_t$ method) with β -actin and GAPDH endogenous controls (demonstrating low variation from microarray analysis), and all real-time reactions carried out in triplicate. Real-time results were analyzed using the SDS software (Applied Biosystems, v.2.2.1), with automatic computation of baseline and threshold fluorescence levels. Gene expression C_t values below that of endogenous controls were discarded from analyses and outlier removal was performed in cases where the standard deviation of C_t values exceeded 0.3 cycles. Student's *t*-tests and Pearson's correlations (between microarray and real-time comparative fold changes) were employed in statistical analysis (SPSS, v.12.0, Chicago, IL, USA). The genes chosen for validation were selected based on their involvement in stress response, signal transduction and neurotransmission, and on the strength of findings from microarray analysis.

Results

No significant differences were observed in terms of age ($C = 35.3 \pm 11.5$; $S = 35.1 \pm 9.0$; $SMD = 36.5 \pm 12.3$), pH ($C = 6.5 \pm 0.3$; $S = 6.3 \pm 0.3$; $SMD = 6.5 \pm 0.4$) and PMI ($C = 23.7 \pm 5.8$; $S = 24.3 \pm 4.5$; $SMD = 24.1 \pm 6.5$) between the groups (Table 1). The effect of age and PMI on quality control parameters like β -actin and GAPDH 5'/3' ratios and the number of present cells was evaluated. No significant correlation was observed between PMI and any of the quality control variables in our sample in all the regions studied. Figure 1 shows the correlations between pH and PMI with 5'/3' ratios for β -actin and GAPDH in hippocampus. Similar relationships were also observed in the other limbic areas.

Initial filtering in the amygdala, which consisted of selecting genes called 'present' (according to MAS 5.0) in at least 75% of the subjects in at least one group, resulted in 15 007 genes that were included in the analysis. The ANOVA and *post hoc* analysis resulted in 182 differentially expressed genes. Among these, 127 genes were significant for the SMD-C comparison, 51 for the S-C comparison and 33 for the SMD-S comparison (Figure 2). The intersections show the number of significant genes common for two comparisons. The PCA plot and the clustered image map show the degree of discrimination between the groups using the differentially expressed genes (Figure 3a and b). Gene ontology analysis based on the differentially expressed genes revealed an over-representation of genes implicated in the regulation of transcription and in nucleic acid metabolism in the amygdala (Table 2). For instance, X-box binding protein 1, a transcription factor gene whose expression was shown previously to be implicated in bipolar disorder,⁴⁸ was upregulated in the two suicide groups and even more among the suicides without

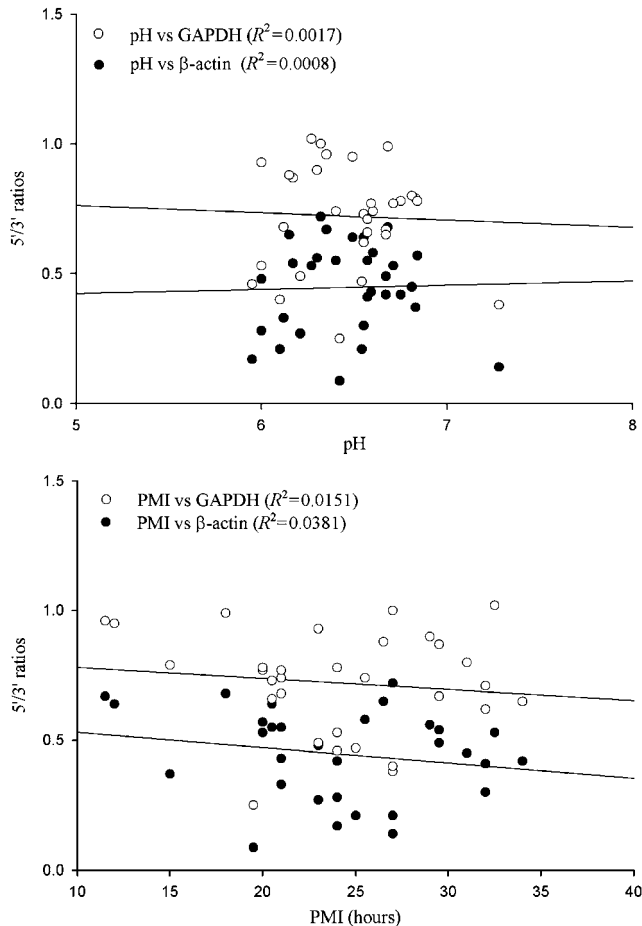


Figure 1 Graphical representation of the correlation between pH and PMI with 5'/3' ratios for β -actin and GAPDH in hippocampus. Similar relationships are observed in the other limbic areas.

major depression (Table 3). Also, several genes playing a role in the regulation of second messenger systems were differentially expressed in the amygdala, among these, there were several protein kinase and protein phosphatases genes, as well as cAMP-dependent kinases (Table 3).

In the hippocampus, 14 537 genes passed the initial filtering and were included in the analysis. A total of 429 genes were differentially expressed between the three groups, 35 for the SMD-C comparison, 120 for the S-C comparison and 359 for the SMD-S comparison (Figure 2). It is noteworthy that in contrast to the other regions, in the hippocampus the vast majority of genes were significantly different between the two groups of suicides, with and without major depression. Accordingly, the hierarchical clustering analysis showed the biggest separation between the two groups of suicides and the clustered image map clearly shows two groups of genes differentially expressed between the two suicide groups (Figure 4a). Also, in the PCA, the 20 genes contributing most to the first component of the PCA are differentially expressed between these two groups, as reflected by

their separation in the two-dimensional space (Figure 4b). Gene ontology analysis revealed an overrepresentation of genes implicated mainly in the regulation of transcription and in nucleic acid-binding and metabolism (Table 2). Overrepresented ontology categories corresponded to several kinases, phosphatases, one phosphodiesterase and one adenylate cyclase (ADCY8), all implicated in intracellular signaling cascades and second messenger systems (Table 2). Several genes implicated in neurotransmission were also altered in the hippocampus such as the genes coding for the multiple coiled-coil GABABR1-binding protein the inositol 1,4,5-triphosphate receptor type 1 the cannabinoid receptor 2 (CNR2) and the leptin receptor. Finally, three genes coding for proteins involved in the biosynthesis and catabolism of polyamines were also differentially expressed in the hippocampus: SSAT2, spermine synthase, and ornithine aminotransferase-like 1 (OATL1).

Analysis in BA24 was conducted on 14 556 genes after the initial filtering and revealed 84 genes as differentially expressed between the three groups. *Post hoc* analysis identified 52 genes as differentially expressed for the SMD-C comparison, 20 genes for the S-C comparison and 27 genes for the SMD-S comparison (Figure 2). A good separation and clustering of the groups was observed with the PCA and the hierarchical clustering analysis using the differentially expressed genes (Figure 5a and b). Gene ontology analysis of the differentially expressed genes revealed an overrepresentation of binding and binding-related functions such as RNA and nucleic acid binding, as well as enzymatic regulation (Table 2). Interestingly, the β -site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1) known to play an important role in neurodegeneration and in Alzheimer disease⁴⁹ was downregulated in the depressed suicide group when compared with controls, suggesting a possible role for this enzyme in the neurobiology of depression. Two gabaergic receptors were also differentially expressed in opposite ways in BA24. Although the γ -aminobutyric acid (GABA) A receptor, α -1 (GABRA1) was highly downregulated among the depressed suicides versus the controls, the GABA A receptor, β -1 (GABRB1) showed an important upregulation for that same comparison. Suicides without major depression showed intermediate levels, suggesting possible quantitative effects on the phenotype.

After filtering, 15 032 genes were included in the analysis for BA29, 83 of which were differentially expressed according to the defined criteria. More specifically, 19 genes were significant for the SMD-C comparison, 40 for the S-C comparison and 40 for the SMD-S comparison (Figure 2). Differentially expressed genes used for the PCA and the hierarchical clustering were able to discriminate the groups, which showed a good separation and an accurate clustering (Figure 6a and b). Gene ontology analysis was performed with the differentially expressed genes, but only 69 genes were fully annotated and

Table 1 Demographic and clinical characteristics of the subjects included in the analysis

Group	Age	PMI	pH	Cause of death	DSM-IV (6 months diagnosis)	Toxicological findings
C	32	26.5	6.80	Cardiac arrest		
C	31	24	5.95	Cardiac arrest	Alcohol dependence	
C	19	32	6.55	Car accident		
C	47	12	6.49	Cardiac arrest	Alcohol abuse	
C	30	30	6.37	Cardiac arrest		
C	28	27	6.32	Car accident		
C	41	24	6.00	Myocardial infarction		
C	31	29.5	6.67	Car accident		
C	46	19.5	6.42	Myocardial infarction		
C	21	24	6.42	Cardiac arrest		
C	27	20.5	6.55	Cardiac arrest		
C	51	15	6.83	Car accident	Alcohol dependence	Alcohol
C	55	24	6.75	Car accident		
S	33	18	6.68	Hanging		
S	38	23	6.00	Hanging	Alcohol dependence, cocaine dependence	Alcohol
S	21	21	6.59	Asphyxiation	Alcohol dependence	Alcohol
S	31	32.5	6.27	Hanging		
S	29	26.5	6.15	Hanging		
S	36	25	6.54	Hanging		
S	51	21	6.12	Self inflicted gun shot	Alcohol dependence	
S	42	27	6.10	Carbon monoxide		
SMD	22	11.5	6.35	Hanging	MDD, alcohol dependence	Alcohol, cocaine
SMD	19	29.5	6.17	Hanging	MDD	
SMD	53	29	6.30	Hanging	MDD, alcohol dependence	
SMD	42	21	6.40	Drowning	MDD	
SMD	45	20.5	6.57	Self inflicted gun shot	MDD	
SMD	39	25.5	6.60	Hanging	MDD	
SMD	49	32	6.57	Hanging	MDD, alcohol abuse	
SMD	26	34	6.67	Hanging	MDD	Cocaine
SMD	40	22	6.96	Hanging	MDD	
SMD	39	19	6.00	Overdose	MDD	
SMD	26	21.5	6.50	Carbon monoxide	MDD, alcohol abuse, cocaine dependence	Cocaine
SMD	35	31	6.81	Hanging	MDD, alcohol dependence	
SMD	53	14	6.45	Carbon monoxide	MDD	
SMD	53	33.5	6.78	Hanging	MDD	
SMD	18	27	7.28	Carbon monoxide	MDD	
SMD	22	20	6.71	Hanging	MDD	
SMD	40	23	6.21	Hanging	MDD, alcohol dependence	
SMD	28	20	6.84	Hanging	MDD, alcohol dependence	Alcohol

Abbreviations: SMD, suicide with major depression; MDD, major depressive disorder.

were included in the enrichment analysis. Probably, owing to the small number of genes, only two ontology terms were significant in BA29 corresponding to organismal movement and protein metabolism. One of these metabolic genes, the SSAT1, was differentially expressed between the two suicide groups.

Because some of the subjects in the three groups had a history of substance dependence or abuse, possibly implicating more chronic substance-related gene expression changes, whereas other subjects had negative history of substance-related disorders, but had a positive toxicological result for alcohol or cocaine (Table 1), which may be associated to more acute substance-related gene expression alterations, we performed an ANOVA to control for these

two different effects on our gene-expression findings. The majority of genes differentially expressed remained significant following this analysis, suggesting that the most of our findings are more directly related to depression and suicide. Table 3 shows the corrected *P*-values (**P*) for genes of interest. Some genes (Table 4) were significantly affected ($P \leq 0.01$) by the history of dependence/abuse of substance (amygdala = 2; hippocampus = 8; BA24 = 0; BA29 = 2) and presence according to the toxicology screening (amygdala = 4; hippocampus = 4; BA24 = 1; BA29 = 1). However, owing to the limited number of subjects per category, further analysis will need to be done in a larger sample to investigate the role played by those genes in substance abuse and intoxication.

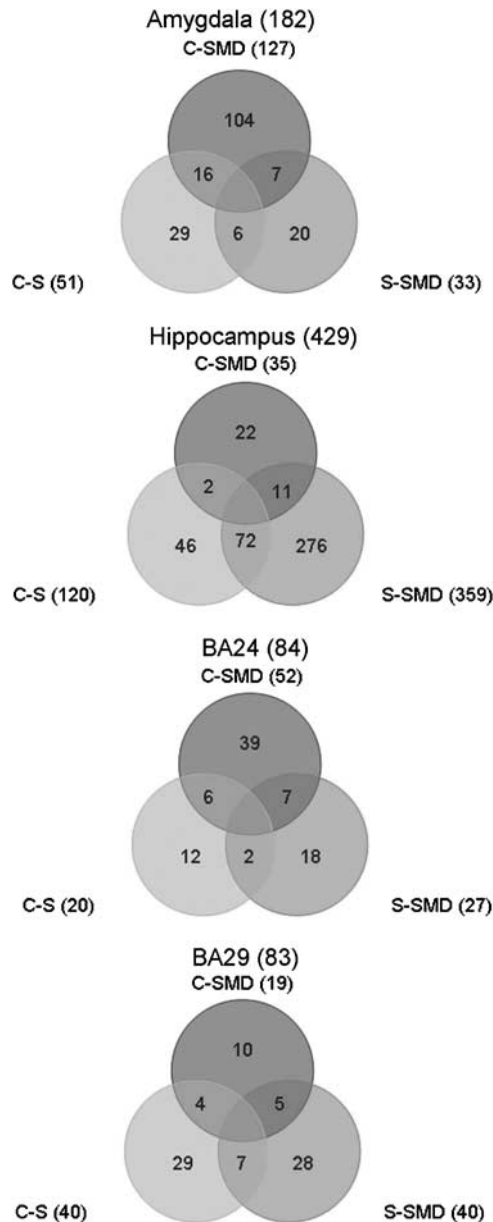


Figure 2 Venn diagrams showing the number of genes identified as differentially expressed and the overlap of genes between the different comparisons in the amygdala, hippocampus, BA24 and BA29. The intersections of the circles indicate the number of genes in common between contrasts.

Similarities in terms of common differentially expressed genes were explored between the three regions. Few similarities in terms of differentially-expressed genes between the regions were observed (amygdala–hippocampus (one gene), amygdala–BA24 (zero genes), amygdala–BA29 (two genes) hippocampus–BA24 (zero genes), hippocampus–BA29 (two genes) and BA24–BA29 (two genes)). This is however not surprising in the case of such areas, which are for the most part functionally and neuroanatomically distinct. More overlap was seen for instance in a

previous study by our group exploring expression in cortical regions.⁵⁰

Some overlap was also observed between the SMD-S and S-C comparisons, suggesting that these genes are likely to be involved in suicide-related processes independently of underlying depressive psychopathology. In the amygdala, for instance four genes were observed in common for those comparisons (MAX, MYEF2, YWHAB and GRIPAP1) whereas only one was observed in BA24 (CIRBP) and three in BA29 (RGN, TDE1 and CKLFSF1). However, in the hippocampus, a total of 72 genes were found in common among which several implicated in signal transduction (ARHGEF7, MAP3K5, RAP2A, RAPH1 and RREB1) and the CNR2.

Evaluation of the microarray fold changes was performed in independent samples collected from adjacent tissue from that used for the microarray assay in a total of 11 controls, seven non-depressed suicides and 13 depressed suicides using real-time PCR. The genes selected for reverse-transcriptase polymerase chain reaction (RT-PCR) validation were chosen based on known function and the significance of microarray findings. Real-time analyses confirmed primary group-level changes in expression for all seven genes selected for further study from the initial set of significant genes: SSAT1 (BA29), SSAT2 (hippocampus), OATL1 (hippocampus), SYT4 (hippocampus), ADCY8 (hippocampus), APLP2 (amygdala) and BACE1 (BA24). The precision as measured by agreement between results of replicate real-time runs was high (mean ΔC_t SD=0.13), with 3% of samples showing a ΔC_t SD in excess of 0.3 cycles. The fold changes at the validation stage ranged from 1.11 to 1.62-fold, and several of these changes exceeded those from the microarray analysis (ADCY8 and SSAT2). For four of the seven genes, the strongest fold change resulted from the S-SMD comparison: OATL1 (microarray FC=1.50, RT FC=1.19), SSAT1 (microarray FC=1.45, RT FC=1.19), SSAT2 (microarray FC=-1.36, RT FC=-1.57), and SYT4 (microarray FC=-1.69, RT FC=-1.11). The BACE1 gene showed reduced expression in the SMD group when compared to the controls (C): BACE1 (microarray FC=-1.32, RT FC=-1.14). Finally, two genes were confirmed as overexpressed in the non-depressed suicide when compared to the controls: ADCY8 (microarray FC=1.46, RT FC=1.62) and APLP2 (microarray FC=1.35, RT FC=1.33). Overall, as seen in other studies using microarrays and brain tissue,⁵¹ correlations between relative fold changes seen using microarray and real-time analyses for the seven genes were good (mean R=0.377, s.d.=0.179). All genes apart from OATL1 and BACE1 demonstrated significant approach-wise correlations in calculated fold changes at the $P<0.05$ level, across all groups. The gene-to-gene variation in the magnitude of fold changes and correlations is likely owing to replication at the tissue rather than RNA level, and the use of an overlapping, but non-identical sample set for confirmation.

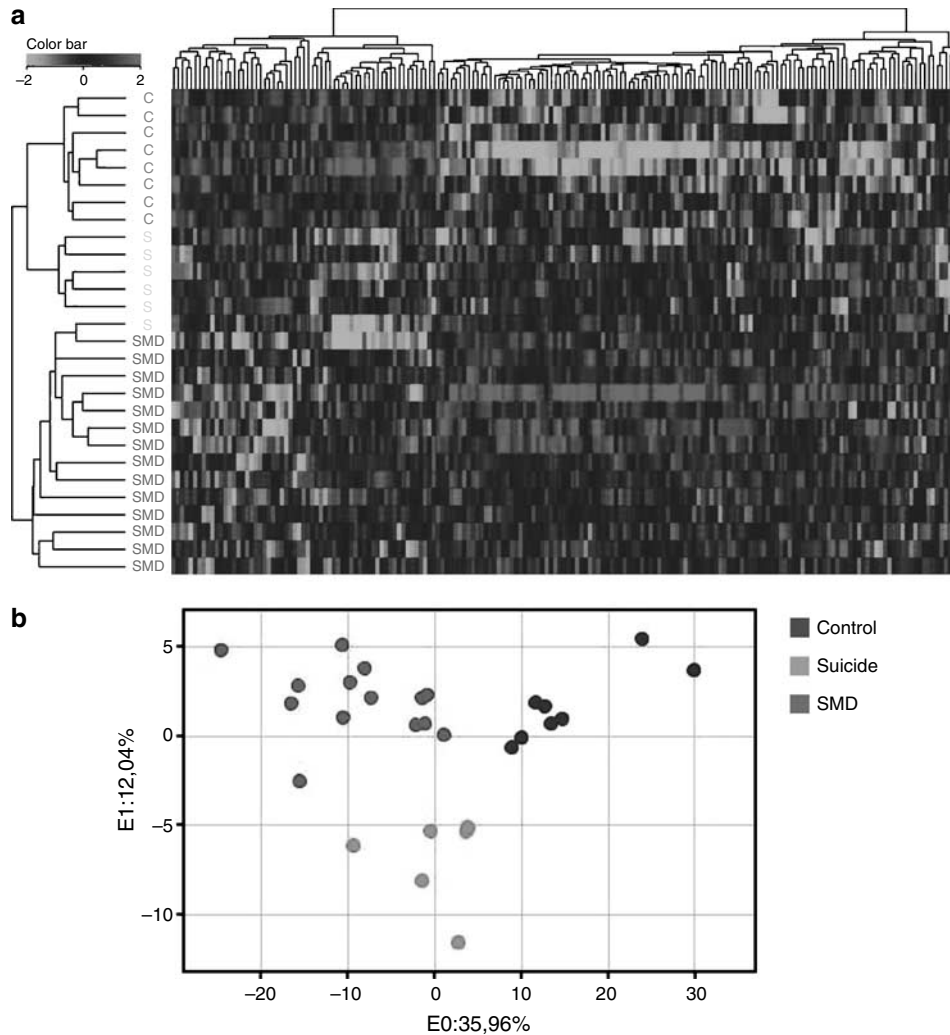


Figure 3 Amygdala gene expression patterns. **(a)** Clusters image map (CIM) of the differentially expressed genes in the amygdala. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate downregulated expression, whereas red spectrum colors indicate upregulated expression. **(b)** PCA based on the differentially expressed genes. The color and intensity indicate direction and level of change: blue spectrum colors indicate downregulated expression, whereas red spectrum colors indicate upregulated expression (online version only).

Discussion

Microarray analysis was performed in four limbic areas of suicides with and without major depression and psychiatrically normal controls. The vast majority of expression changes were observed in the amygdala (182 probe sets), and particularly, in the hippocampus (429 probe sets, of 761 across all four regions). In contrast, fewer differentially expressed genes were observed in the two cingulate cortex regions (BA24 = 84; BA29 = 83). Selected genes for validation with an alternative method (RT-PCR) showed consistently correlated signals with the results obtained in the microarray analysis, supporting the ability of the study design to identify candidate molecular target that may be involved in the neurobiology of major depression and suicide.

We used a design that allowed us to control for the presence of suicide (suicides with major depression versus suicides without major depression) and therefore, to identify processes that are more likely to be implicated in major depression. As such, in our design, the identification of processes exclusively attributed to suicide is confounded by the presence of psychopathology. Nevertheless, by having a normal control group, we also had the ability to identify molecular processes that may be related to suicidality independently of major depression (S-C comparison). Having a comparison group with multiplex developmental disorder (MDD) that did not die by suicide would have allowed us to fully separate effects attributed to suicide. However, for such a group to be comparable, it would have been necessary to include subjects who were affected with MDD before

Table 2 Enriched gene ontology terms calculated using the differentially expressed genes per region

Category	Term	Genes	Total DAVID genes	%	P-value
<i>Amygdala</i>					
Biological process	Intracellular signaling cascade	11	135	8.1	0.028
Biological process	Cell proliferation	11	135	8.1	0.049
Biological process	Transcription	17	135	12.6	0.038
Biological process	Regulation of biological process	22	135	16.3	0.035
Biological process	Nucleotide and nucleic acid metabolism	23	135	17	0.038
Biological process	Cellular process	43	135	31.9	0.043
Biological process	Metabolism	46	135	34.1	0.046
Molecular function	Adenyl nucleotide binding	12	135	8.9	0.045
Molecular function	Protein binding	23	135	17	0.000
<i>Hippocampus</i>					
Biological process	Regulation of transcription, DNA-dependent	35	348	10.1	0.034
Biological process	Regulation of transcription	35	348	10.1	0.048
Biological process	Regulation of metabolism	38	348	10.9	0.035
Biological process	Regulation of biological process	53	348	15.2	0.009
Biological process	Nucleotide and nucleic acid metabolism	58	348	16.7	0.003
Molecular function	Zinc ion binding	29	348	8.3	0.011
Molecular function	Transition metal ion binding	33	348	9.5	0.005
Molecular function	DNA binding	40	348	11.5	0.012
Molecular function	Ion binding	43	348	12.4	0.020
Molecular function	Metal ion binding	43	348	12.4	0.020
Molecular function	Protein binding	44	348	12.6	0.001
Molecular function	Nucleic acid binding	63	348	18.1	0.001
Molecular function	Catalytic activity	95	348	27.3	0.002
<i>BA24</i>					
Molecular function	Enzyme regulator activity	7	70	10	0.003
Molecular function	RNA binding	8	70	11.4	0.000
Molecular function	Protein binding	11	70	15.7	0.030
Molecular function	Nucleic acid binding	15	70	21.4	0.036
Molecular function	Binding	34	70	48.6	0.008
<i>BA29</i>					
Biological process	Organismal movement	5	66	7.6	0.032
Biological process	Protein metabolism	14	66	21.2	0.036

DAVID genes correspond to the total number of unique DAVID annotated genes. The percentage represents the number of differentially expressed genes belonging to a given category over the total number of DAVID annotated genes.

death, or at least in the last 6 month. Collecting a group that would be demographically comparable with the suicide group and that died while affected with MDD is operationally challenging.

The large number of differentially expressed genes in the hippocampus was mainly owing to differences between the two groups of suicides. As processes related to the neurobiology of suicide are controlled for when comparing both suicide groups, the observed expression differences in this region are probably more specifically associated to biological mechanisms related to major depression. This supports recent findings indicating an important role of the hippocampus in depression and in the antidepressant response to pharmacotherapy.^{52,53} Also, altered size and impaired function of the hippocampus have been found in a number of recent clinical imaging studies of major depression (for a meta-analysis see Campbell⁵⁴). Furthermore, expression changes in genes

implicated in the second messenger systems, such as kinases, phosphatases and the adenylate cyclase 8 gene (ADCY8) were observed in the hippocampus, supporting previous evidence of synaptic plasticity alterations in mood disorders^{55,56} and in antidepressant response beyond the neurotransmitter and receptor level.⁵⁷ Recent molecular studies in post-mortem brains have also revealed alterations in second messenger systems in hippocampi of suicides and depressed suicides.^{36,37,58,59}

Few studies have explored global expression changes between groups of suicides and psychiatrically normal controls, some have however focused on a particular psychiatric diagnosis, such as bipolar disorder or schizophrenia, and included within these groups subjects that died by suicide.^{60–67} Sibille *et al.*⁵¹ recently compared expression patterns in BA9 and BA47 of depressed suicides versus psychiatrically normal controls matched on the basis of sex, age,

Table 3 Selected differentially expressed genes in amygdala, hippocampus, BA24 and BA29

Probe set	Gene title	Symbol	Cytoband	C	S	SMD	*P	SMD-C			S-C			S-SMD		
								P	FC	P	FC	P	FC	P	FC	
Amygdala																
208704_x_at	Amyloid beta (A4) precursor-like protein 2	APLP2	11q23-q25	1058	1415	1225	0.013	0.06	1.16	0.00	1.35	0.06	1.16	0.06	1.16	
203006_at	Inositol polyphosphate-5-phosphatase, 40 kDa	INPP5A	10q26.3	204	288	265	0.008	0.01	1.29	0.00	1.41	0.33	1.10	0.33	1.10	
225278_at	Protein kinase, AMP-activated, β -2 non-catalytic subunit	PRKAB2	1q21.1	219	275	305	0.001	0.00	1.40	0.01	1.27	0.01	-1.10	0.01	-1.10	
225011_at	Protein kinase, cAMP-dependent, regulatory, type II, alpha	PRKAR2A	3p21.3-p21.2	368	419	486	0.001	0.00	1.32	0.06	1.14	0.02	-1.16	0.02	-1.16	
206687_s_at	Protein tyrosine phosphatase, non-receptor type 6	PTPN6	12p13	51	31	36	0.012	0.01	-1.43	0.01	-1.61	0.34	-1.12	0.34	-1.12	
200670_at	X-box binding protein 1	XBP1	22q12.1	74	99	92	0.022	0.01	1.23	0.01	1.33	0.42	1.08	0.42	1.08	
Hippocampus																
206811_at	Adenylate cyclase 8 (brain)	ADCY8	8q24	52	76	64	0.013	0.10	1.20	0.00	1.46	0.06	1.22	0.06	1.22	
226463_at	ATPase, H + transporting, lysosomal 42kDa, V1 subunit C, isoform 1	ATP6V1C1	8q22.3	580	425	609	0.002	0.66	1.05	0.01	-1.36	0.00	-1.43	0.00	-1.43	
204311_at	ATPase, Na + /K + transporting, β -2 polypeptide	ATP1B2	17p13.1	294	486	339	0.009	0.37	1.14	0.00	1.66	0.02	1.46	0.02	1.46	
206586_at	Cannabinoid receptor 2 (macrophage)	CNR2	1p36.11	99	143	99	0.006	0.90	-1.01	0.00	1.44	0.01	1.46	0.01	1.46	
213275_x_at	Cathepsin B	CTSB	8p22	296	236	364	0.002	0.07	1.26	0.22	-1.24	0.00	-1.56	0.00	-1.56	
227961_at	Cathepsin B	CTSB	8p22	564	358	650	0.006	0.26	1.25	0.16	-1.49	0.00	-1.86	0.00	-1.86	
203710_at	Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	3p26-p25	229	201	347	0.004	0.01	1.56	0.55	-1.11	0.00	-1.73	0.00	-1.73	
211323_s_at	Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	3p26-p25	110	101	155	0.014	0.02	1.42	0.64	-1.08	0.01	-1.54	0.01	-1.54	
227095_at	Leptin receptor	LEPR	1p31	94	91	67	0.015	0.01	-1.44	0.67	-1.04	0.02	1.38	0.02	1.38	
238600_at	Multiple coiled-coil GABABR1-binding protein	MARLIN1	4p16.1	420	303	476	0.000	0.08	1.14	0.04	-1.43	0.00	-1.63	0.00	-1.63	
205669_at	Neural cell adhesion molecule 2	NCAM2	21q21.1	54	65	45	0.013	0.09	-1.23	0.14	1.19	0.01	1.46	0.01	1.46	
229463_at	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	9q22.1	368	277	487	0.002	0.07	1.29	0.10	-1.42	0.00	-1.83	0.00	-1.83	
236095_at	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	9q22.1	209	188	264	0.002	0.01	1.27	0.33	-1.11	0.00	-1.41	0.00	-1.41	
227820_at	Ornithine aminotransferase-like 1	OATL1	Xp11.3-p11.23	150	196	135	0.020	0.41	-1.13	0.02	1.33	0.01	1.50	0.01	1.50	
236300_at	Phosphodiesterase 3A, cGMP-inhibited	PDE3A	12p12	104	131	91	0.011	0.26	-1.15	0.04	1.27	0.00	1.46	0.00	1.46	
203680_at	Protein kinase, cAMP-dependent, regulatory, type II, beta	PRKAR2B	7q22	207	240	318	0.001	0.00	1.53	0.15	1.17	0.02	-1.30	0.02	-1.30	
227278_at	Protein phosphatase 1A	PPM1A	14q23.1	519	379	579	0.001	0.13	1.11	0.02	-1.42	0.00	-1.57	0.00	-1.57	
235061_at	Protein phosphatase 1K (PP2C domain containing)	PPM1K	4q22.1	215	194	319	0.016	0.03	1.58	0.90	-1.03	0.00	-1.62	0.00	-1.62	
208617_s_at	Protein tyrosine phosphatase type IVA, member 2	PTP4A2	1p35	476	672	527	0.016	0.33	1.12	0.01	1.44	0.01	1.29	0.01	1.29	
225272_at	Spermidine/spermine N1-acetyltransferase 2	SSAT2	17p13.1	636	509	694	0.005	0.35	1.08	0.03	-1.26	0.00	-1.36	0.00	-1.36	
202043_s_at	Spermine synthase	SMS	Xp22.1	109	136	154	0.011	0.00	1.40	0.05	1.24	0.23	-1.13	0.23	-1.13	
205551_at	Synaptic vesicle glycoprotein 2B	SV2B	15q26.1	936	775	1055	0.008	0.12	1.13	0.13	-1.22	0.00	-1.39	0.00	-1.39	
225721_at	Synaptopodin 2	SYNPO2	4q26	179	258	175	0.012	0.90	-1.02	0.02	1.47	0.00	1.69	0.00	1.69	
223529_at	Synaptotagmin IV	SYT4	18q12.3	1579	1353	2359	0.001	0.03	1.45	0.24	-1.16	0.00	-1.69	0.00	-1.69	
225204_at	T-cell activation protein phosphatase 2C	TA-PP2C	12q24.11	333	231	331	0.006	0.88	-1.02	0.01	-1.46	0.01	-1.43	0.01	-1.43	
225213_at	T-cell activation protein phosphatase 2C	TA-PP2C	12q24.11	377	228	347	0.002	0.36	-1.09	0.01	-1.71	0.00	-1.57	0.00	-1.57	
BA24																
24335_s_at	β -site APP-cleaving enzyme 1	BACE1	11q23.2-q23.3	339	303	253	0.015	0.00	-1.32	0.37	-1.10	0.02	1.20	0.02	1.20	
206678_at	γ -aminobutyric acid (GABA) A receptor, alpha 1	GABRA1	5q34-q35	301	244	175	0.020	0.00	-1.72	0.29	-1.21	0.06	1.42	0.06	1.42	
207010_at	γ -aminobutyric acid (GABA) A receptor, beta 1	GABRB1	4p12	510	478	714	0.007	0.00	1.42	0.72	-1.05	0.00	-1.49	0.00	-1.49	
229773_at	Synaptosomal-associated protein, 23 kDa	SNAP23	15q15.1	83	63	78	0.013	0.40	-1.07	0.00	-1.33	0.03	-1.25	0.03	-1.25	
BA29																
221482_s_at	Cyclic AMP phosphoprotein, 19kDa	ARPP-19	15q21.2	1000	1197	873	0.014	0.13	-1.22	0.04	1.21	0.01	1.48	0.01	1.48	
242482_at	Protein kinase, cAMP-dependent, regulatory, type I, α	PRKARIA	17q23-q24	107	80	86	0.014	0.02	-1.24	0.01	-1.33	0.38	-1.07	0.38	-1.07	
203455_s_at	Spermidine/spermine N1-acetyltransferase	SSAT	Xp22.1	230	279	199	0.008	0.16	-1.18	0.08	1.23	0.00	1.45	0.00	1.45	

For a gene to be considered as differentially expressed it had to have an ANOVA $P \leq 0.01$ and for a given contrast a fold change/ P -value combination of at least 1.3-fold change in both direction and $P \leq 0.01$. Corrected P -values ($*P$) are shown after controlling for the possible confounder effect of substance dependence/abuse. Significant comparisons (FC > 1.3 | and $P \leq 0.01$) are in bold.

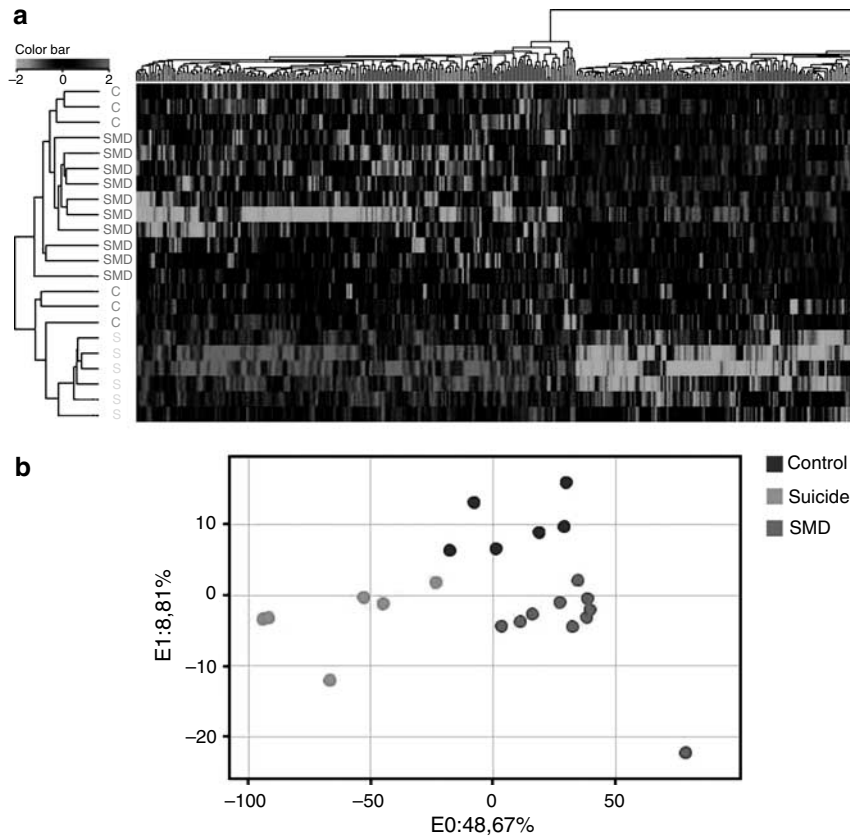


Figure 4 Hippocampus gene expression patterns. **(a)** CIM of the differentially expressed genes in the hippocampus. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate downregulated expression, whereas red spectrum colors indicate upregulated expression. **(b)** PCA based on the differentially expressed genes. The color and intensity indicate direction and level of change: blue spectrum colors indicate downregulated expression, whereas red spectrum colors indicate upregulated expression (online version only).

PMI, and race. Despite the number of transcripts investigated they observed no significant evidence of differences in gene expression that correlated with major depression and suicide. In limbic regions, structural abnormalities have been observed consistently in the past decades in psychiatric disorders^{68–70} and recent expression studies in suicides have confirmed altered expression of genes implicated in neurotransmission and intracellular signalling in some limbic areas^{33,35–37,58,71–73} in agreement with the observed results in the present paper.

One potential limitation of human brain microarray studies is the possible confounding effect on gene expression by psychoactive drugs. In spite of the presence of psychopathology in suicides, most of the cases used in this study were not actively treated before death. Accordingly, only two cases had a history of antidepressant treatment. This is consistent with data from the literature that indicates that most suicide completers were not properly treated before death.^{74,75} In our sample, as expected for suicides, several subjects had a history of substance dependence/abuse, primarily involving alcohol. This was the case in the two suicide groups, and to a lesser

degree, also in the control group. However, after controlling for the history of alcohol and/or cocaine dependence/abuse or the presence of substance intoxication as per toxicological results, the group effect on the expression differences observed for most genes remained significant, suggesting that the gene expression patterns observed are not owing to the effect of alcohol or cocaine. Nevertheless, for certain genes, a specific effect of acute or chronic substance exposure was observed. The effect alcohol and cocaine on the expression of those genes in the context of suicide and depression should be further explored.

One of the challenges with microarray studies is to process the large amounts of information generated. Differentially expressed genes were fully annotated using electronic databases (NetAffix) and further explored using gene ontology and enrichment algorithms (DAVID), which constitutes an interesting way to summarize and functionally explore microarray data despite some problems concerning vague higher functional classifications. In our study, we implemented stringent significance criteria in the identification of differentially expressed genes (minimum

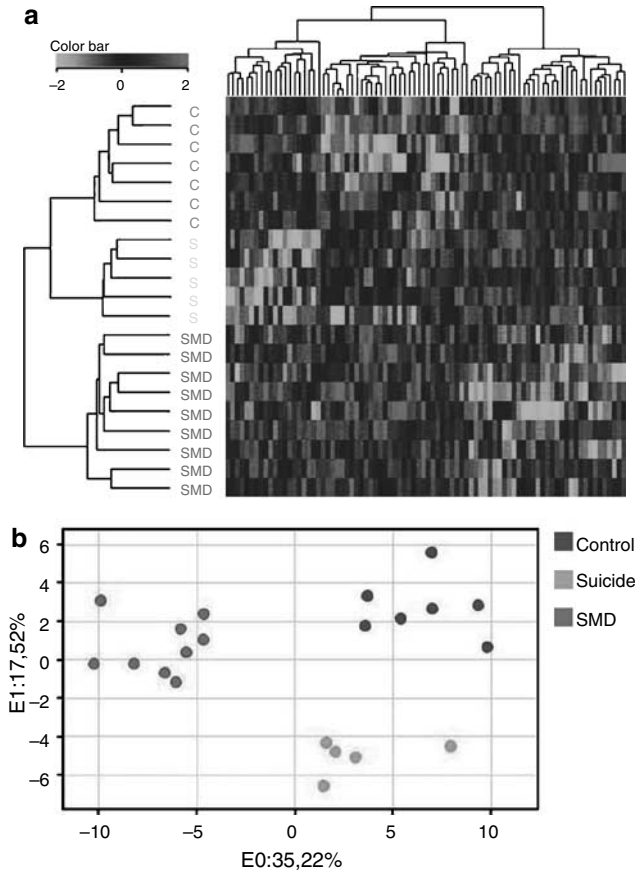


Figure 5 BA24 gene expression patterns. (a) Clusters image map (CIM) of the differentially expressed genes in BA24. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, whereas red spectrum colors indicate up-regulated expression. (b) PCA based on the differentially expressed genes. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, whereas red spectrum colors indicate up-regulated expression (online version only).

1.3-fold change, ANOVA and *t*-test *P*-values < 0.01) to reduce the complexity of the data and enrich for genes, which are biologically relevant to suicide and depression. The combination of the ANOVA and subsequent *t*-tests is commonly referred to as Fisher protected least significant difference (LSD) test. In the case of gene expression studies of complex traits such as psychiatric disorders, it has been suggested to use less stringent criteria in the initial analysis stages,⁷⁶ and the use of only *t*-tests or Fisher protected LSD test has been commonly accepted in psychiatric brain expression studies^{50,64,65} as well as in other research areas.⁷⁷

Multiplicity is always a concern when performing microarray analysis and many correction procedures have been developed to address it such as the Bonferroni-method or false discovery rate-based methods.⁷⁸ However, these methods as commonly

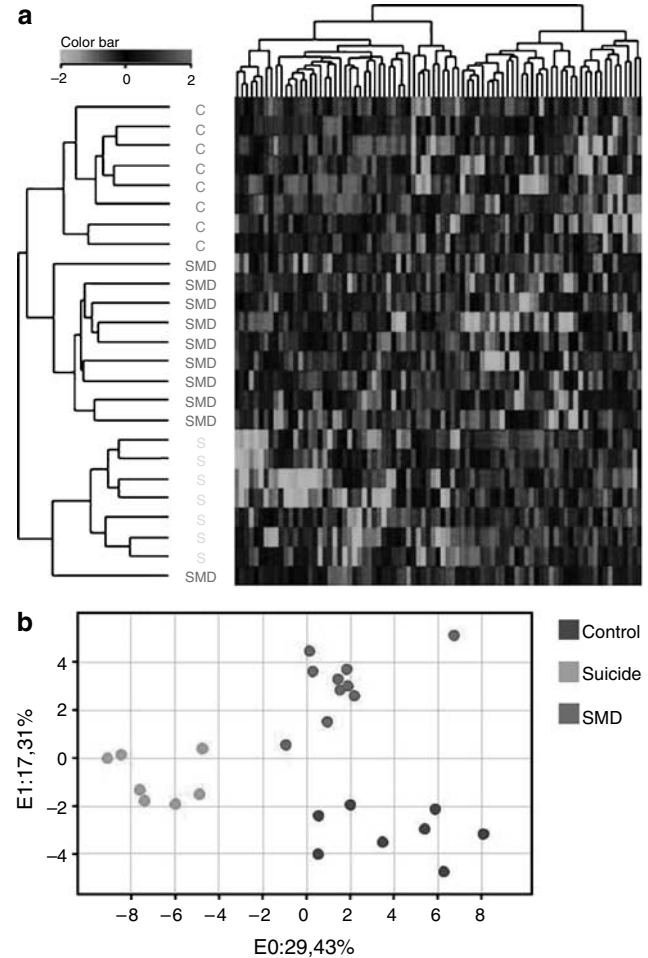


Figure 6 BA29 gene-expression patterns. (a) CIM of the differentially expressed genes in BA29. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, whereas red spectrum colors indicate up-regulated expression. (b) PCA based on the differentially expressed genes. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, whereas red spectrum colors indicate up-regulated expression (online version only).

used, assume independence of the hypotheses being tested.⁷⁹ This is far from reality in the context of gene expression in brain tissue, where there is extensive multicollinearity between probe-sets as a result of genes in pathways, or those implicated in a particular molecular function, which can be regulated in a coordinated manner. For instance, many researchers have found not genes but families of genes or pathways as being implicated in psychiatric disorders in the past.^{62,64,65,76,80–85} One additional consideration for this research was the distinction between depressed and non-depressed suicides, permitting the evaluation of depression-specific effects on gene expression separately from suicide-specific effects.

The observation of a disproportionate number of genes identified as differentially expressed between

Table 4 Genes significantly affected by the history of substance dependence or abuse (chronic substance effects) and positive substance intoxication as per toxicological result for alcohol or cocaine (acute substance effects) in the amygdala, hippocampus, BA24 and BA29

Probeset	Gene title	Symbol	Chromosome	Substance effects														
				Groups					Chronic					Acute				
				df	F	P*	df	F	P	df	F	P	df	F	P			
<i>Amygdala</i>																		
219855_at	Nudix (nucleoside diphosphate linked moiety X)-type motif 11	NUDT11	Xp11.22	2	11.525	0.000	1	8.874	0.007	1	6.104	0.021						
223497_at	KIAA1411	KIAA1411	6q12-q13	2	10.511	0.001	1	2.388	0.136	1	12.133	0.002						
225919_s_at	Chromosome 9 open reading frame 72	C9orf72	9p21.2	2	3.574	0.044	1	9.640	0.005	1	0.771	0.389						
226406_at	Chromosome 18 open reading frame 25	C18orf25	18q21.1	2	9.360	0.001	1	0.103	0.752	1	16.885	0.000						
239425_at	Full length insert cDNA clone ZC34E11		1	2	11.306	0.000	1	1.846	0.187	1	10.178	0.004						
239437_at	Transcribed locus		6	2	7.152	0.004	1	5.937	0.023	1	14.912	0.001						
<i>Hippo</i>																		
202780_at	3-oxoacid CoA transferase 1	OXCT1	5p13.1	2	14.357	0.000	1	5.016	0.039	1	10.543	0.005						
204258_at	Chromodomain helicase DNA binding-protein 1	CHD1	5q15-q21	2	11.834	0.001	1	7.676	0.013	1	10.944	0.004						
210176_at	Toll-like receptor 1	TLR1	4p14	2	12.077	0.001	1	1.749	0.204	1	9.115	0.008						
212262_at	Quaking homolog, KH domain RNA binding (mouse)	QKI	6q26-27	2	20.340	0.000	1	10.686	0.005	1	6.355	0.022						
219269_at	Hypothetical protein FLJ21616	FLJ21616	8p21.1	2	10.775	0.001	1	12.002	0.003	1	1.707	0.209						
223880_x_at	Chromosome 20 open reading frame 24	C20orf24	20q11.23	2	12.852	0.000	1	9.985	0.006	1	8.341	0.010						
227450_at	Hypothetical protein FLJ32115	FLJ32115	12p12.3	2	17.002	0.000	1	10.596	0.005	1	10.475	0.005						
228549_at	KIAA0792 gene product	KIAA0792		2	9.882	0.001	1	9.793	0.006	1	0.415	0.528						
229966_at	Ewing sarcoma breakpoint region 1	EWSR1	22q12.2	2	11.193	0.001	1	10.866	0.004	1	3.943	0.063						
235366_at	Zinc-finger protein 10	ZNF10	12q24.33	2	11.702	0.001	1	13.546	0.002	1	3.837	0.067						
91816_f_at	Ring-finger and KH domain-containing 1	RKHD1	19p13.3	2	11.926	0.001	1	8.789	0.009	1	1.095	0.310						
<i>BA24</i>																		
52837_at	KIAA1644 protein	KIAA1644		2	8.426	0.003	1	0.309	0.586	1	9.014	0.008						
<i>BA29</i>																		
204544_at	Hermansky-Pudlak syndrome 5	HPS5	11p14	2	13.681	0.000	1	14.823	0.001	1	9.748	0.005						
241876_at	Mdm4, transformed 3T3 cell double minute 4, p53-binding protein (mouse)	MDM4	1q32	2	12.587	0.000	1	11.142	0.003	1	1.647	0.214						

Corrected *P*-values (**P*), after controlling for the possible confounder effect of substance dependence/abuse and *P*-values associated with the history of substance dependence or abuse (Chronic) and positive toxicological result (Acute) for alcohol or cocaine are shown.

the suicide groups in the hippocampus is clearly reflected in the PCA, with the top 10 eigen values for the first component arising from genes differentially expressed between the depressed and non-depressed suicide groups. This contrast between suicide groups is consistent with the prominent role of the highly plastic hippocampus in depression and stress response,^{20,54} discriminating between depressive and non-depressive states for a subset of differentially expressed genes. A gene ontology analysis of probes that were significantly differentially expressed between the two suicide groups in the hippocampus reveals a strong overrepresentation of factors involved in transcriptional regulation and metabolism, similar to the result including all comparisons in the hippocampus, but with a much higher degree of significance. The enrichment of factors involved in metabolic processes may outline a differential stress response between the suicide groups, whereas the preponderance of transcriptional regulators may act on diverse networks of additional genes, enhancing the apparent difference between these two groups in the hippocampus. In addition, the gene expression pattern in the hippocampus may be broadly related to long-term changes caused by a recurrent depressive state, this being contrary to changes brought about by a more transient and excitable state for the non-depressed suicides. A more focused and critical investigation of the hippocampus in further suicide victims will help to discriminate between these possibilities.

Amongst the genes validated in the four limbic regions, several play fundamental roles in generalized neurotransmission, as in the case of APP-like protein 2 (APLP2), the absence of which in knockout mice is associated with a reduction in both density and number of docked vesicles at the active zone.⁸⁶ Both APLP2 and APP are processed by the product of the also identified BACE1 gene,⁸⁷ exposing a pathway considered of primary importance in the pathogenesis of Alzheimer's disease. The apparent upregulation of synaptotagmin 4 (SYT4, a presynaptic calcium-sensor and regulator of synaptic release) gene expression in the hippocampus of depressed suicide victims parallels findings in the SYT4 knockout mouse, which displays reduced levels of anxiety and depression-like behavior, as well as altered short-term plasticity (CA1) and hippocampal-dependent memory,^{88,89} and the upregulated hippocampal expression of ADCY8 in non-depressed suicides compared with controls integrates with a convincing body of research demonstrating perturbed cAMP signaling in bipolar disorder,⁹⁰ depression⁹¹ and suicide.^{92–94} These genes constitute interesting avenues for further investigation.

Several gabaergic system genes were identified as differentially expressed in this investigation, in both BA24 and hippocampus, drawing attention to a possible gabaergic dysfunction in the limbic system of suicidal and depressive individuals. Two genes are of particular interest, GABA A receptor, α -1 (GABRA1)

and GABA A receptor, β -1 (GABRB1) (Table 3), as GABA receptors mRNA levels have been associated previously with depression and suicidality.⁹⁵ Inhibitory neurotransmission in the mammalian brain is mainly accomplished by gabaergic neurons, responsible for the release of GABA.⁹⁶ GABA neurotransmission modulates the activity of noradrenergic, dopaminergic and serotonergic systems.⁹⁶ Clinical data have pointed to an alteration in gabaergic neurotransmission in mood disorders and more specifically in major depression.⁹⁷ Thus, cerebrospinal fluid and plasma studies have observed altered levels of GABA in depressed patients. Also, SSRIs are known to increase GABA levels in the brain of depressed patients.⁹⁸ Finally, our results also confirm the recent findings of another group concerning gene expression abnormalities in GABA signal transmission in the cerebral cortex of subjects, who had suffered from depression and more specifically GABA(A) and GABA(B) alterations in individuals who died by suicide suggesting their potential role in suicidality.⁶² Our findings together with previous studies clearly point to an alteration of gabaergic neurotransmission in suicide and major depression in the context of suicide and offers new potential pharmacological targets for the treatment of such complex disorders.

Real-time PCR has been widely used in the confirmation of findings from microarray studies owing to its high sensitivity and precision. However, although these advantages may be significant, a number of technique and analysis-specific parameters capable of greatly influencing the final result have recently been discussed,⁹⁹ including factors such as quality of the template, the reverse-transcription step, selection of endogenous controls, and the data analysis approach employed. Current research has emphasized the need for multiple control genes for relative quantification¹⁰⁰ owing to the inconsistency in expression of what were considered previously as 'housekeeping' genes. Rigorous sample quality control and two reference genes were used in this study, with the real-time fold changes and correlations between microarray and real-time data supporting the validation of the most significant results from the microarray component. The correlation between microarray and real-time data could at first sight seem modest, but one has to take into account the fact that this RT-PCR assays were not carried out in the same RNA sample, but rather on RNA extracted from additional samples from the same subjects obtained from adjacent tissue to that used for in the original microarray experiments. This is different from what is usually performed in most microarray studies, which use the same RNA sample. By doing our procedure, we are not only validating the microarray result, but also the intra subject reproducibility. Finally, because the purpose of the RT-PCR was solely a technical replication of the results obtained in the microarray, the biological replication was obtained by using multiple samples from different subjects, a slightly

less conservative criteria for RNA quality was used for the RT-PCR than for the microarray. As our RT-PCR results correlate positively with the microarray data, even when using adjacent samples from the same subjects for both experiments, we can consider with an acceptable degree of confidence that issues related to RNA integrity were unlikely to be responsible for our results.

Microarray experiments should be regarded as screening assays capable of shedding light onto biological processes involved in complex conditions such as those investigated in this study. As such, the sets of differentially expressed genes obtained in each region constitute interesting targets for future studies focused on suicide and major depression. To our knowledge this investigation represents the first gene expression study on suicides and depressed suicides examining genome-wide alterations in limbic brain regions. The expression profile in the hippocampus underlies the importance of this structure in major depression in the context of suicide, and implicates a fundamental role for alterations of the second messenger systems. Additionally, our results confirm previous evidence pointing to alterations of gabaergic neurotransmission in suicide and major depression, offering new avenues in the study, and possibly treatment, of such complex conditions. Further studies are warranted to confirm these results not only at the mRNA level, but also at the protein and functional level, as well as the link between molecular alterations with symptoms and risk factors associated with suicide and with major depression.

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