

# Molecular Characterization of Suicide by Microarray Analysis

FUAD G. GWADRY, ADOLFO SEQUEIRA, GLENN HOKE, JARLATH M.H. FFRENCH-MULLEN, AND GUSTAVO TURECKI\*

Several lines of evidence support the idea that individuals who commit suicide have a certain biological predisposition, part of which is given by genes. Studies investigating genetic factors increasing suicide predisposition have been limited by current knowledge of the suicide neurobiology and have typically investigated one or a few genes at a time, whereas it is anticipated that several genes account for the total genetic variance mediating suicide. This review focuses on the advantages and the interest of using the microarray technology to investigate the neurobiology of suicide and discusses, by means of a data analysis example, the possible methodological problems and bioinformatic strategies that should be employed in order to separate the signal from the large amount of background noise, which is usually generated in such studies. Microarray expression studies and related platforms are promising tools to gain better insight into the neurobiology of suicide.

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**KEY WORDS:** brain; microarrays; mRNA; psychiatric disorders; suicide; genetics

## INTRODUCTION

The World Health Organization (WHO) reports that suicide accounts for almost 2% of the world's deaths [WHO, 2000]. In most of the developed

world, suicide is among the 10 top leading causes of death for individuals of all ages [Mao et al., 1990; Statistics, 1997], and is the leading cause of death for males younger than 40 years of age [Mao et al., 1990; Statistics, 1997]. Suicide is a complex behavior that is most probably the result of the interaction of several different factors. Over the last decades, it has become increasingly clear that individuals who commit suicide have a certain predisposition [Blumenthal and Kupfer, 1990; Mann, 1998; Mann, 2002], which is, among other factors, mediated by the genetic make up. Accordingly, there is growing evidence from genetic epidemiological studies supporting the role of genes in the predisposition toward suicide [Tsuang, 1983; Egeland and Sussex, 1985; Roy, 1993; Brent et al., 1996, 2002, 2003; Johnson et al., 1998; Turecki, 2001; Kim et al., 2003; Qin et al., 2002]. These studies suggest that genetic predisposition to suicide is given by different genetic factors from those conferring susceptibility to psychiatric disorders [Brent et al., 1996; Statham et al., 1998; Glowinski et al., 2001; Turecki, 2001; Fu et al., 2002]. In recent years, a growing number of molecular

studies have been carried out to investigate candidate genes that may be involved in suicidal behavior [Anguelova et al., 2003]. Because of the nature of the phenotype investigated, family-based mapping studies have been difficult to carry out. Instead, the focus has been on case-control association designs using a candidate gene approach. Beyond well-known methodological shortcomings of candidate-gene association studies [Risch and Merikangas, 1996; Risch, 2000], this approach is also limited by the choice of candidate genes, which has been primarily based on current knowledge about the neurobiology of suicide, and therefore, the vast majority of candidate-gene association studies conducted thus far have focused on genes coding for components of the serotonergic system [Anguelova et al., 2003]. Another limitation of this approach is that such studies have been most commonly investigating one gene at a time, whereas it is anticipated that several genes account for suicide's total genetic variance [Turecki, 2001].

Current methodology allows circumventing some of these limitations. Brain microarray expression studies provide parallel monitoring of several

Fuad G. Gwady is a mathematician who has been working on bioinformatics and chemoinformatic solutions for the drug discovery process. Currently he is CSO with G-BioChem Solutions, a scientific research firm.

P. Adolfo Sequeira, M.Sc. is a molecular biologist who is currently completing his Ph.D. training in the genetics of mood disorders and suicide at the McGill Group for Suicide Studies, McGill University.

Glenn Hoke is past Vice President of Research & Development at Gene Logic, Inc., and currently he is President and CSO at Dyad Pharmaceutical Corp.

Jarlath M.H. ffrench-Mullen is a neuroscientist and currently scientific director of the Central Nervous System (CNS) program at GeneLogic, Inc.

Gustavo Turecki M.D., Ph.D. is a psychiatrist and CIHR scholar who directs the McGill Group for Suicide Studies at McGill University.

\*Correspondence to: Gustavo Turecki, M.D., Ph.D., McGill Group for Suicide Studies, Douglas Hospital, McGill University, 6875 LaSalle Blvd., Verdun, QC H4H 1R3, Canada. E-mail: gustavo.turecki@mcgill.ca  
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thousand to virtually all genes, and as such, they give a “snapshot” of brain gene activity prior to death [Bunney et al., 2003]. They are particularly useful to identify new candidate systems and to gain new insight into biological mechanisms mediating suicide. There has been an exponential use of microarray technology in health research over the last years. In psychiatry, microarray studies are still at incipient stages and only more recently such studies have begun to emerge. This review will discuss the advantages of using microarray studies to investigate the neurobiology of suicide together with possible methodological problems and bioinformatic strategies that should be employed in order to separate the signal from the large amount of background noise that is usually generated.

## BRIEF OUTLINE OF MICROARRAY TECHNOLOGY

Based on hybridization of oligonucleotide probes mounted on high density arrays to a target nucleotide sequence, microarrays are basically of two different kinds: those based on direct deposition of DNA [Schena et al., 1995], or spotted arrays, and those based on direct synthesis of probes by photolithography [Lockhart et al., 1996]. The latter is the method used by Affymetrix and has been the most commonly used microarray technology in expression studies. Currently available Affymetrix chips for expression analysis (HG U133) allow for monitoring of over ~45,000 transcripts, which represent most of the Human Genome (see [www.affymetrix.com](http://www.affymetrix.com)). Several 25-mer oligonucleotides are used for detection of each gene. Affymetrix uses what it calls the perfect match/mismatch probe strategy, consisting of synthesizing probes that are perfect matches to the target sequence and pairing these with an equivalent number of mismatch probes which are perfectly identical to the perfect matches if not by a changed nucleotide on position 13. This strategy allows the quantitation and subtraction of signals caused by non-specific cross-hybridiza-

tion and then controlling for background noise. These microarrays are used to hybridize biotin-labeled nucleotides synthesized from target mRNA molecules, which after hybridization are fluorescently stained and scanned.

## USING POSTMORTEM BRAIN TISSUE IN MICROARRAY EXPRESSION STUDIES

In central nervous system (CNS) microarray studies, total RNA is isolated from selected postmortem brain tissue using standard RNA extraction methods such as those using phenol-guanidine isothiocyanate and then purified with RNA purification columns. Published human postmortem studies have primarily used frozen tissue and the utilization of formalin-fixed tissue in microarray studies remains to be better investigated. Some preliminary studies suggest that although studies using fixed tissue may perform well, its use may be limited, particularly by the observed increase in result variability [Karsten et al., 2002].

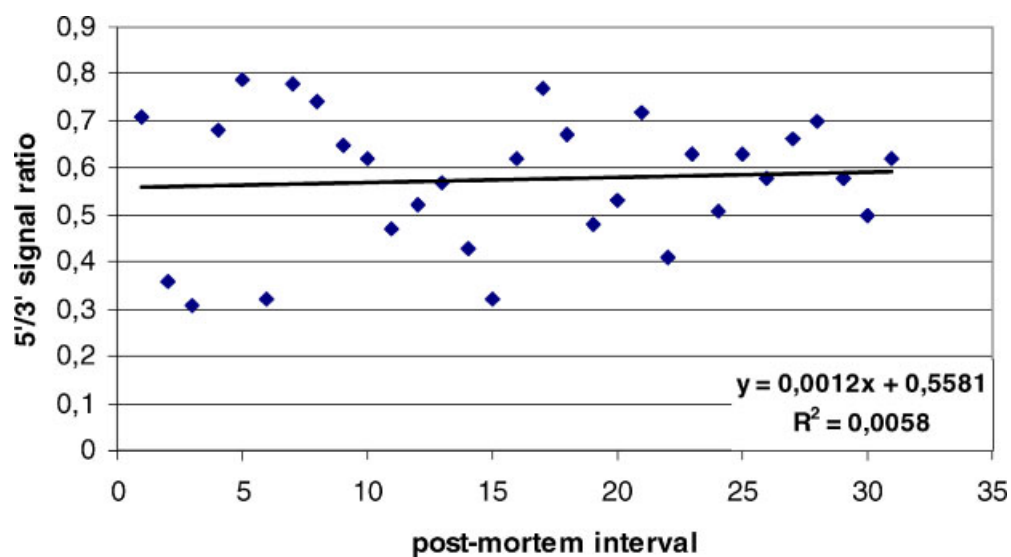
Following RNA extraction, samples are inspected after gel electrophoresis for integrity appearance and spectrophotometrically scanned from 220 to 330 nm to control for RNA quality based on A260/A280 and 28S/18S ratios. RNA quality control is an essential part of microarray expression studies in general, but in the context of postmortem brain studies, this is a particularly important step. Accordingly, a number of variables may influence RNA quality and increase between-sample variability, including demographic factors, direct cause of death, agonal factors, postmortem delay, tissue pH, post-autopsy tissue handling, dissection procedures and tissue storage conditions [Albrecht and Yanagihara, 1979; Perry et al., 1982; Wester et al., 1985; Johnson et al., 1986; Perrett et al., 1988; Kobayashi et al., 1990; Burke et al., 1991; Barton et al., 1993; Leonard et al., 1993; Harrison et al., 1995; Bahn et al., 2001; Cummings et al., 2001; Yasojima et al., 2001; Preece and Cairns, 2003; Li et al., 2004; Tomita

et al., 2004]. However, although not all studies agree, of these variables, agonal factors are thought to more directly influence RNA integrity through a larger influence on tissue pH [Albrecht and Yanagihara, 1979; Harrison et al., 1995; Tomita et al., 2004].

In addition to pre- and postmortem factors, RNA integrity and quality may be experimentally controlled for using a number of different techniques [Ryan et al., 2004]. However, when using Affymetrix microarrays, the most important procedures to control for RNA quality are based on the analysis of signal ratios between 3' and 5' probes from  $\beta$ -actin and GAPDH housekeeping genes. Because cDNA synthesis is based on poly(dT) priming, samples with high level of RNA degradation will have low 5' signal and thus, low 5'/3' ratios. Therefore, this ratio gives an indication of the integrity of the starting RNA sample and efficiency of the cDNA and cRNA syntheses. As there is no single threshold cutoff proposed to assess sample quality, it is important to assess 5'/3' ratios of individual samples and exclude outliers. Figure 1 plots 5'/3' ratios against postmortem intervals (PMI) in a series of samples from our expression studies, indicating that there is no influence of PMI on RNA quality.

## ANALYSIS EXAMPLE

To illustrate the ability and power of microarray brain gene expression studies to better understand the neurobiology of suicide, we present as an example the analysis of tissue obtained from the orbital gyri (BA11), of suicide completers who had a history of major depressive disorder (N=6) as compared to normal controls (N=6). Details on psychological autopsies used for diagnostic procedures are reported elsewhere [Kim et al., 2003]. All subjects were male Caucasians of French-Canadian origin. Subjects were matched on the basis of age and postmortem interval. The mean ages for the controls and the depressed suicides were respectively  $36 \pm 14$  and  $37 \pm 9$  years, while the mean PMI for the controls and depressed suicides were respectively



**Figure 1.** Relationship between RNA-QC and the postmortem interval (PMI) in a series of postmortem brain samples. RNA-QC is a summary of the 5'/3' ratios for  $\beta$ -actin and GAPDH. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

$23 \pm 5$  and  $25 \pm 7$  hr. Independent analysis of experimental and demographic parameters revealed no effect of age ( $P = 0.87$ ) and postmortem interval ( $P = 0.56$ ). All subjects, including controls, died suddenly without a prolonged agonal state or protracted medical illness.

Sample preparation and processing, hybridization to the Human Genome U133 GeneChip set (arrays A and B) and normalization were performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA). Total RNA samples were spectrophotometrically scanned from 220 to 330 nm. RNA extractions utilized in the current study had a minimum A260/A280 ratio of  $>1.9$ . Acceptable A260/A280 ratios fall in the range of 1.8–2.1; ratios below 1.8 indicate possible protein contamination; ratios above 2.1 indicate presence of degraded RNA, truncated cRNA transcripts, and/or excess free nucleotides. 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).

GeneChip analysis was performed based on the Affymetrix GeneChip Manual (Affymetrix) with Microarray

Analysis Suite (MAS) 5.0, Data Mining Tool (DMT) 2.0, and Microarray Database software. All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 100. Description of the Affymetrix normalization is available on the internet ([http://www.affymetrix.com/support/technical/technotes/statistical\\_reference\\_guide.pdf](http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf)).

## DATA ANALYSIS

Expression data were stored in the BioExpress<sup>TM</sup> CNS Suite (Gene Logic, Inc., Gaithersburg, MD) and analyzed using Avadis software (Strand Genomics Pvt. Ltd., <http://www.strandgenomics.com>).

## SELECTION OF ARRAYS

Principal component analysis (PCA) was used to quickly identify outlier arrays. Parameters examined to assess hybridized array quality included the following: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, consistent  $\beta$ -actin and GAPDH 5'/3' signal ratios and consistent detection of BioB and BioC hybridization spiked controls. Quality control parameters for the example data

set were similar between the controls and the depressed suicides (data not shown). Table I reports the overall distribution for each of the parameters. A description of the Affymetrix quality control parameters is available at [http://www.affymetrix.com/support/downloads/manuals/data\\_analysis\\_fundamentals\\_manual.pdf](http://www.affymetrix.com/support/downloads/manuals/data_analysis_fundamentals_manual.pdf).

Overall, the samples retained for analysis displayed high 5'/3' ratios (Table I). A ratio close to one indicates low or absent mRNA degradation. No correlation between sample variability in terms of RNA QC and PMI was observed (Fig. 1), indicating a high RNA integrity for these postmortem brain samples and no influence of PMI on RNA quality.

## SELECTION OF INITIAL GENE SET

As the false discovery rate is a function of the number of tests, we reduced the number of genes being tested. We selected genes for analysis from the  $\sim 45,000$  on the array on the basis of "Present Calls" by MAS 5.0. Under MAS 5.0, Affymetrix evaluates the perfect match (PM) and mismatch (MM) intensities for a probe set. They look at whether the PM is significantly

**TABLE I. Quality Control Parameters for Brain Sample Microarrays From the Orbital Gyrus, Brodmann Area (BA) 11 (C, n = 6; SD, n = 6)**

	RawQ	Scale factor	% Present calls	Actin 5'/3' ratio	GAPDH 5'/3' ratio
<b>U133A</b>					
Mean + SEM	2.66 + 0.19	1.36 + 0.10	40.86 + 1.02	0.54 + 0.05	0.74 + 0.04
Max	4.62	1.81	48.14	0.79	0.93
Min	2.14	0.85	35.47	0.25	0.41
<b>U133B</b>					
Mean + SEM	2.67 + 0.17	2.74 + 0.19	27.25 + 0.82	0.54 + 0.05	0.69 + 0.04
Max	3.89	3.66	31.32	0.78	0.89
Min	1.81	1.52	22.68	0.25	0.35

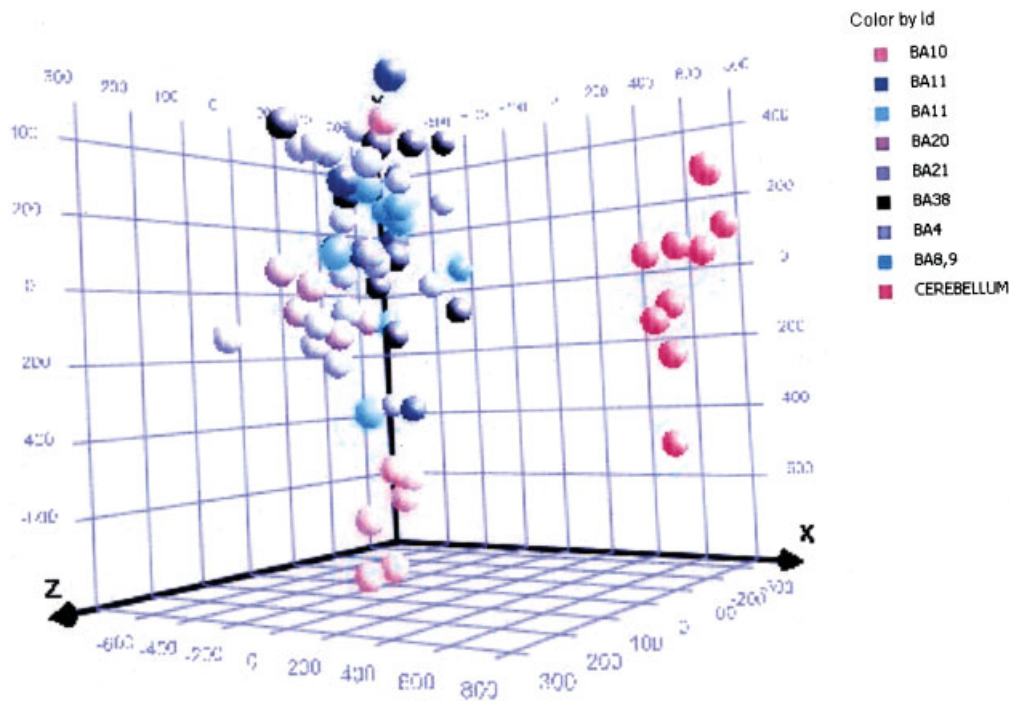
Values were derived from MAS 5.0 array analysis. The lower percent present calls in the B chip compared to the A chip is due to the fact that the B chip contains primary probe sets representing EST clusters. As a result overall signal intensities on the B chip are lower which is reflected by higher scaling factors. Note that  $\beta$ -actin and GAPDH signal ratios are consistent across chips

above the MM and have developed a *P*-value to describe the significance. Given certain *P*-value limits, the probe set is called either present, marginal, or absent. In the current study, for a gene to be included, it had to be 75% present (detectable) in at least one of the groups. This resulted in a total of 14,027 genes that were used in the subsequent steps.

### DISCRIMINANT POWER OF POSTMORTEM BRAIN STUDIES

For initial confirmation of the reliability of our microarray analysis, we compared control samples from seven cortical regions (BA10, BA11, BA20, BA21, BA38, BA4, and BA8,9) and the cere-

bellum. PCA of these regions was performed on the ~22,000 genes on the HG-U133A chip (Fig. 2). The spatial discrimination between the cortical and the reference region, the cerebellum, on the first component demonstrates the ability and the sensitivity of brain expression studies to detect region specific functional gene expression patterns.



**Figure 2.** Principal component analysis (PCA) performed based on the ~22,000 genes on the HG-U133A Chip from control samples in eight cortical regions (BA10, BA11, BA20, BA21, BA38, BA4, BA8,9) and the cerebellum.

## DIFFERENTIAL GENE EXPRESSION ANALYSIS

The first step in the analysis was the transformation of the values to a log scale with all subsequent steps performed on the log-transformed values [Nadon and Shoemaker, 2002]. The unpaired *t*-test for two groups was performed for each gene in order to identify differentially expressed genes. Note that this test (asymptotic analysis) relies on the assumption that the expression values for a given gene within each group are normally distributed and that the variances of the normal distributions associated with the two groups are the same. These assumptions may be violated for some of the genes. Permutation analysis methods do not require assumptions about the distribution of the data. In a permutation test the group labels (“depressed-suicides” or “suicides”) are randomly permuted and test statistics are computed for each permutation. The test statistics from the permuted datasets are compared to the test statistic from the “original” dataset, and a *P*-value computed as the proportion of all datasets that have test statistics greater than or equal to the value from the “original” dataset. Table II reports the

number of genes satisfying various *P*-value and fold-change combinations when using asymptotic and permutation analysis, demonstrating that both methods yielded similar results. Using a fold change/*P*-value combination of 1.3 fold change in either direction and a  $P < 0.01$ , 292 of the initial set of 14,026 genes were identified as being differentially expressed. Of these, 73 were up-regulated and 218 were down-regulated in depressed-suicides in relation to controls.

## HIERARCHICAL CLUSTER ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES

Cluster analysis was performed on these 292 genes using average-linkage hierarchical cluster analysis with a correlation metric. The data are presented in the form of color-coded cluster image maps (CIMs) where the color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression (Fig. 3). Both the expression patterns in individuals and genes were clustered. The cluster analysis showed

two distinct clusters corresponding to the two groups. Figure 3B shows a zoomed section of the CIM in Figure 3A, including the serotonergic receptor *5-HT2A* and co-clustering genes. The serotonin (5HT) type 2A receptor (*HRT2A*) gene was up-regulated in depressed-suicides in relation to controls with an  $FC = +1.8$  and  $P = 0.00063$ . Thus, the microarray analysis is consistent with the well documented involvement of the *5-HT2A* gene in suicide and mood disorders [Mann et al., 2001; Turecki, 2001].

## PCA

PCA was also performed on the selected 292 gene set (Fig. 3C). The first three components accounted for 82.01% of the total variance. Components 1, 2, and 3 accounted for 71.54%, 5.87%, and 4.60% of the variance, respectively. The first component illustrates the distinct separation between the depressed-suicides and controls. This spatial discrimination suggests that the disease affected either the extent of co-regulation between genes, or the grouping of co-regulated genes within the depressed-suicides and control groups.

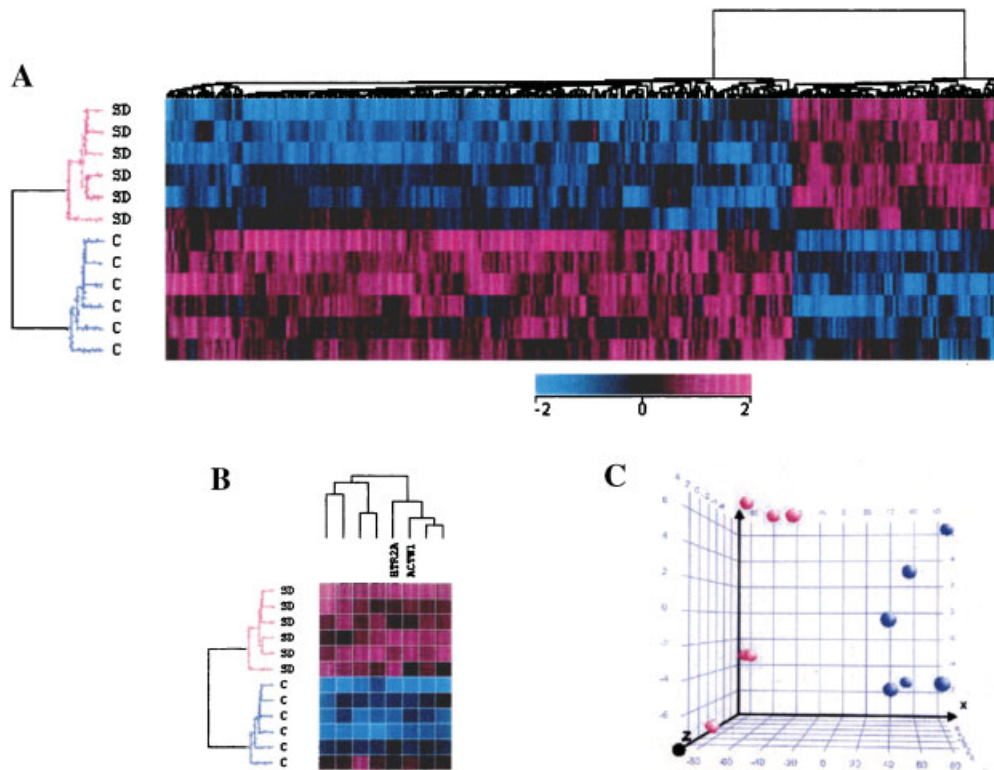
## FUNCTIONAL INTERPRETATION

The analyses according to functional characteristics involves interpretation of the biological meaning of the various groups of genes that have been produced (usually known as gene clusters). The work of the Gene Ontology (GO) Consortium provides a way to address this question. GO organizes genes into hierarchical categories based on biological process, molecular function and subcellular localization. Detailed documentation for the GO is available at the Gene Ontology homepage (<http://geneontology.org>). A gene product can have one or more molecular functions, be used in one or more biological processes, and may be associated with one or more cellular components. Each term is derived from one or more parent terms [Ashburner et al., 2000].

**TABLE II. Differential Expression Analysis Results: The Number of Genes Satisfying Various *P*-Value and Fold-Change Combinations**

	<i>P</i> all	<i>P</i> < 0.05	<i>P</i> < 0.02	<i>P</i> < 0.01	<i>P</i> < 0.005	<i>P</i> < 0.001
<b>a</b>						
FC all	14,026	2,241	1,282	833	493	143
FC > 1.1	8,785	2,239	1,282	833	493	143
FC > 1.5	1,197	864	640	443	292	93
FC > 2	219	189	155	114	83	29
FC > 3	26	23	18	12	9	1
<b>b</b>						
FC all	14,026	2,372	1,417	914	622	276
FC > 1.1	8,785	2,371	1,417	914	622	276
FC > 1.5	1,197	899	680	485	361	165
FC > 2	219	197	165	128	98	54
FC > 3	26	23	21	17	14	6

The *P*-values are computed using (a) asymptotic analysis and (b) permutation analysis. The former computes *P*-values based on normal distribution assumptions while the latter does not rely on this assumption.



**Figure 3.** **A:** Clustered image map (CIM) of the hierarchical cluster analysis of the 292 significantly different genes in the orbital gyrus (BA11). 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, while red spectrum colors indicate up-regulated expression. There are two main clusters of genes co-regulated, 218 were up-regulated and 73 were down-regulated in the depressed suicides in relation to controls. **B:** CIM of a subsample of genes up-regulated among the depressed suicides when compared to the controls. The serotonergic receptor 5-HT2A and the cytoskeleton protein Actinin 1 are examples of genes up-regulated among the depressed suicides. **C:** PCA based on the differentially expressed genes. Blue circles indicate controls, while red circles indicate depressed suicide.

For the 292 genes, the distribution of GO terms between the up- and down-regulated genes depressed-suicides in relation to controls was investigated. The total percentage of genes with ontology terms for biological process, molecular function, and sub-cellular localization is illustrated in Figure 4. The percentages are based on the total number of genes with ontology terms within the up- and down-regulated genes. The total number of genes with GO terms at level 3 for (A) biological process was 153, of which 36 were up-regulated and 117 down-regulated. The total number of genes with GO terms at level 3 for (B) molecular function was 157, of which, 36 were up-regulated and 121 down-regulated. A total of 134 genes had GO terms at level 3 for (C) subcellular

localization, of which, 35 were up-regulated and 99 were down-regulated.

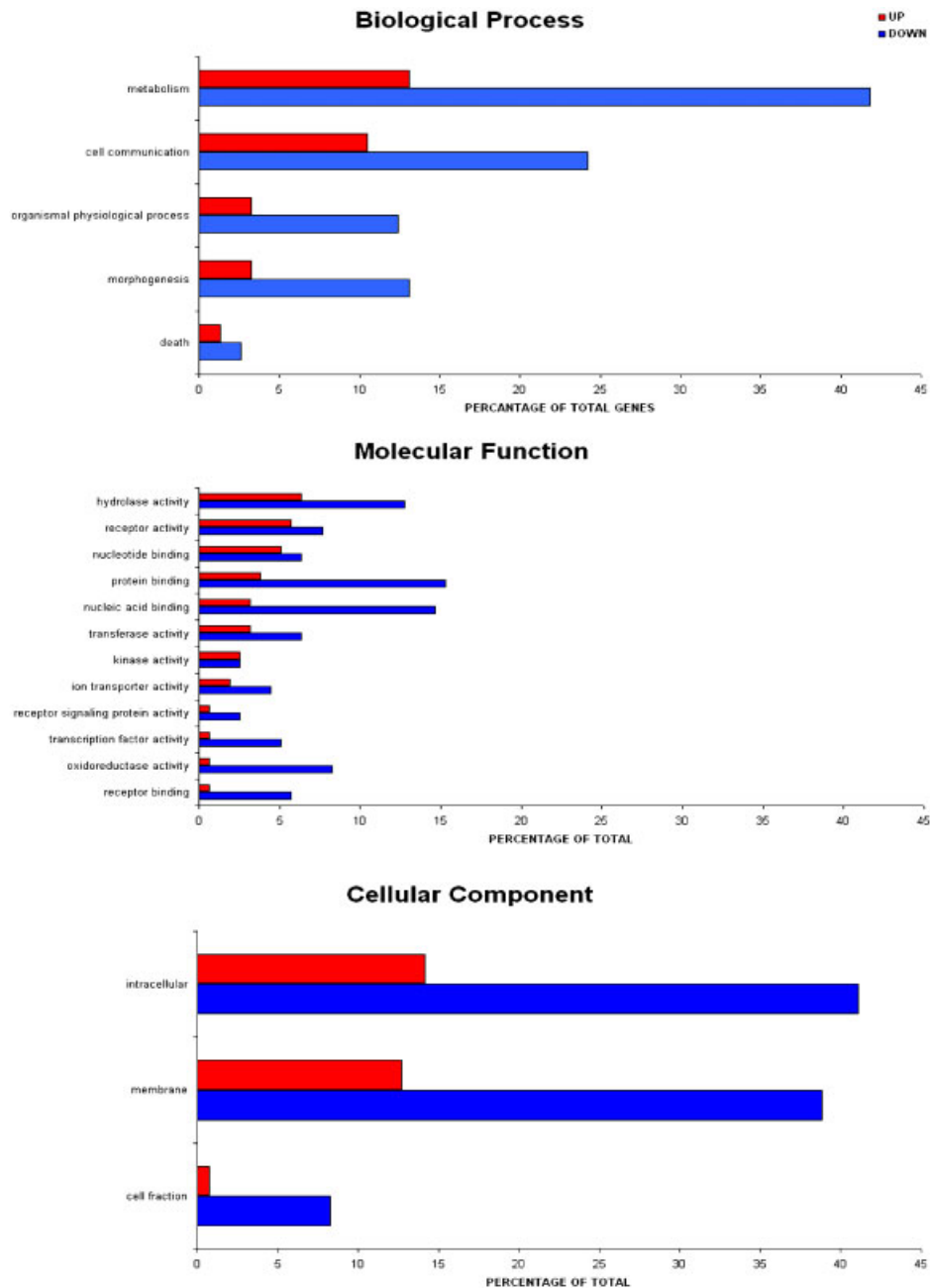
The comparison of the GO annotations between up- and down-regulated genes in depressed suicides versus controls reveals the importance of certain ontology categories in the differences observed between the two groups (Fig. 4). Two molecular categories related to neurotransmission (receptor activity and receptor signalling protein activity), showed important differences between up- and down-regulated genes (Fig. 4).

The serotonergic 2A receptor gene expression (*5-HT2A*), up-regulated among the depressed-suicides when compared to the controls, is an example of the differentially expressed genes implicated in neurotransmission. Figure 5 gives a visual representation of

the Gene Ontology terms as a hierarchical structure in the GO browser for *5-HT2A*.

## DISCUSSION

Microarray expression technology is a useful tool to examine differential gene expression patterns of genes across a broad spectrum of the human genome and as such to identify interesting gene clusters and pathways that may be of relevance to better understand pathophysiological processes involved in the etiology of suicide and related behaviors/psychopathology. Although RNA quality from postmortem brain tissue is a potentially important issue [Albrecht and Yanagihara, 1979; Perry et al., 1982; Wester et al., 1985; Johnson et al., 1986; Perrett et al., 1988; Kobayashi



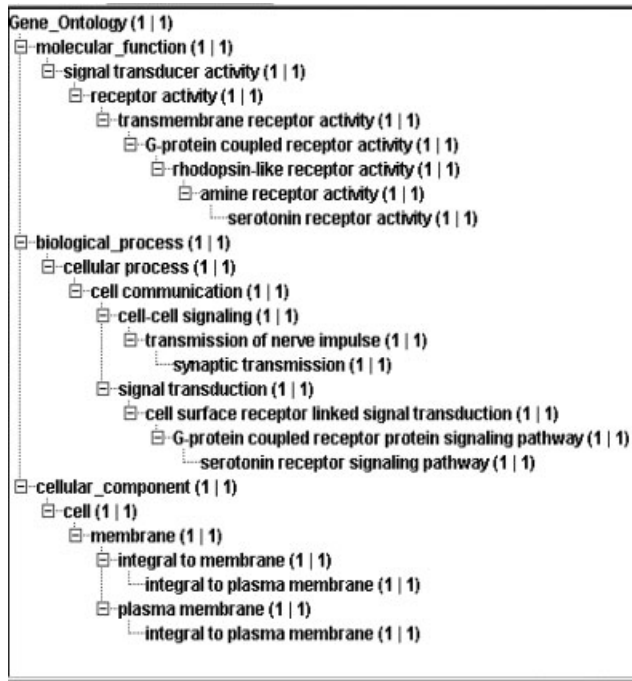
**Figure 4.** Comparison of the Gene Ontology (GO) categories between up-regulated and down-regulated genes using the web based gene-ontology comparison program Fatigo. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

et al., 1990; Burke et al., 1991; Barton et al., 1993; Leonard et al., 1993; Harrison et al., 1995; Bahn et al., 2001; Cummings et al., 2001; Yasojima et al., 2001; Preece and Cairns, 2003; Li et al., 2004; Tomita et al., 2004], the data briefly presented above indicate that when sufficient attention is paid to controlling for RNA quality, brain

expression studies can produce good quality and valid data capable of discriminating between different tissues and phenotypes.

Microarray technologies have become standard tools in many areas of biomedical research. The use of microarrays for gene expression profiling is a powerful tool for screening and identi-

fying genes that may encode proteins involved in molecular mechanisms underlying psychiatric diseases and suicide. In performing gene expression analysis on brain tissues, a critical component is having the ability to easily procure samples from specific regions of the brain, which are of sufficient quantity and quality to support mRNA



**Figure 5.** Visual representation of the GO terms for *5-HT2A*. A GO term is represented as a hierarchical structure in the GO browser.

sample isolation, the subsequent purification of the mRNA, and finally the amplification processes that allows signal detection with microarrays.

While the brain is highly structured with many sub-regions and nuclei, at the cellular level there is even greater complexity. This has led to sample processing involving the use of laser capture microdissection (LCM) to obtain a small tissue sample that is from a defined region and that is comprised of specific cell types. Protocols for isolation and amplification of mRNA derived from LCM samples are currently available and are being used to obtain more disease, and cell type-specific gene expression patterns. Having the ability to inexpensively monitor expression of relevant genes, can provide valuable insight into the disease process and allow for researchers to look towards the cellular biology of abnormal processes.

A growing number of high throughput platforms have become available to monitor the expression of specific gene systems or subsets in a larger number of samples. With the introduction of these platforms, resea-

rchers have the ability to increase the specificity of their assays and confidence level in defining specific genes or pathways that may correlate to abnormal function.

In summary, although microarray expression studies of psychiatric disorders and suicide are just beginning to emerge, they are a valuable and powerful research tool. Together, microarray expression studies and related platforms will help gain better insight into the neurobiology of suicide and may lead to the identification and validation of biological markers of suicidal behavior.

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