Critical Factors in Gene Expression in Postmortem Human Brain: Focus on Studies in Schizophrenia

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Background: *Studies of postmortem human brain are important for investigating underlying pathogenic molecular mechanisms of neuropsychiatric disorders. They are, however, confounded by pre- and postmortem factors. The purpose of this study was to identify sources of variation that will enable a better design of gene expression studies and higher reliability of gene expression data.*

Methods: *We assessed the contribution of multiple variables to messenger RNA (mRNA) expression of reference (housekeeping) genes measured by reverse transcriptase–polymerase chain reaction (RT-PCR) by multiple regression analysis in a large number (N = 143) of autopsy samples from the hippocampus and white and grey matter of the dorsolateral prefrontal cortex (DLPFC) of patients with schizophrenia and normal control subjects.*

Results: *The strongest predictor of gene expression was total RNA quality. Other significant factors included pH, postmortem interval, age and the duration of the agonal state, but the importance of these factors depended on transcript measured, brain region analyzed, and diagnosis. The quality of RNA obtained from the DLPFC white matter was also adversely affected by smoking.*

Conclusions: *Our results show that normalization of expression data of target genes with a geometric mean of multiple housekeeping genes should be used to control for differences in RNA quality between samples. The results also suggest that accurate assessment of other confounding factors and their inclusion as regressors in the analysis is critical for obtaining reliable and accurate quantification of mRNA expression.*

Key Words: Schizophrenia, smoking, RNA integrity, postmortem studies, brain bank, hippocampus, prefrontal cortex

Schizophrenia is a psychiatric disorder with a strong heritable component. Recent linkage and association studies provide new evidence for the involvement of multiple genes in schizophrenia (Craddock et al 2005; Harrison and Weinberger 2005). However, none of the candidate genes have yet been unequivocally confirmed, and their functional roles in the pathophysiology of the disease are unclear. Conclusive evidence of causative genes will require demonstration that variation in genetic sequence predicts biological effects in brain that bear on the pathogenesis of the disorder. For this, studies of postmortem human brain are critically important, because they allow the investigation of underlying pathogenic cellular and molecular mechanisms in a native context that other techniques, such as in vivo imaging, animal models, or cell culture systems cannot readily address. One promising approach is to determine whether genotype and diagnosis predict changes in messenger RNA (mRNA) and protein levels of candidate genes (Bray et al 2005; Egan et al 2004; Law et al 2006; Lipska et al 2006; Xu et al 2005), thereby hinting at their functional role in the disorder.

Collecting and characterizing human brains has remained methodologically challenging (Hulette 2003; Ravid et al 1992), and measurements of gene expression in postmortem tissue are complicated by numerous confounding factors, including age, gender, pH, postmortem interval, tissue quality, antemortem medication, smoking, manner of death, agonal processes, and various other variables, each of unknown relative effect (Bahn et al 2001; Barton et al 1993; Burke et al 1991; Harrison 1996; Johnston et al 1997; Kingsbury et al 1995; Lewis 2002; Miller et al 2004; Preece and Cairns 2003; Preece et al 2003; Tomita et al 2004). To improve the reliability and accuracy of findings, it is critical to start from a carefully selected, well-characterized brain series. Establishing such a cohort is challenging, because the standards regarding inclusion criteria necessary for rigorous postmortem research are still unclear (Kleinman et al 1995; Lewis 2002).

In this study, we set out to evaluate a set of factors that have been implicated previously (see aforementioned references) as potentially critical for conducting studies of gene expression in postmortem brain samples from patients with schizophrenia and normal control subjects. A large number of autopsy samples allowed us to assess the effects of multiple pre- and postmortem factors on mRNA expression of endogenous reference genes by multivariate analysis. We also estimated statistical power in these cohorts for investigating group differences in gene expression.

Methods and Materials

Subjects

Human brain specimens were collected in the Section on Neuropathology of the Clinical Brain Disorders Branch at the National Institute of Mental Health (NIMH) through the Offices of the Chief Medical Examiner of the District of Columbia and of Northern Virginia, after autopsy, and through tissue donations via funeral homes. Informed consent to study brain tissue was obtained from the surviving next-of-kin for all cases, according to Protocol #90-M-0142 approved by the NIMH/National Institutes of Health Institutional Review Board. A telephone interview with the next-of-kin to gather basic demographic information and medical, substance use, and psychiatric history was conducted within 1 week of donation.

Diagnosis. Postmortem clinical diagnosis was established with previously described methods (Deep-Soboslay et al 2005). Briefly, clinical information was gathered by conducting family interviews with the next-of-kin, consisting of the Structured Clinical Interview for DSM-IV–clinician version (SCID-CV) (First

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et al 1997) and the NIMH psychological autopsy interview adapted in part from Columbia University Psychological Autopsy Interview (Kelly and Mann 1996) and the University of Pittsburgh postmortem interview (D.A. Lewis, unpublished) (see also Deep-Soboslay et al 2005) as well as by psychiatric record reviews with the Diagnostic Evaluation After Death (DEAD) (Zalcman and Endicott 1983). After psychiatric record reviews and postmortem family interviews were completed, brief psychiatric narratives were prepared on each case, summarizing the demographic, clinical, medical, and death information obtained from as many sources as possible (i.e., multiple psychiatric records, police reports, neuropathology reports, medical examiner's information, toxicology screen, postmortem family interview). Each case was then independently reviewed by two board-certified psychiatrists, who arrived at consensus DSM-IV Axis I lifetime diagnoses (American Psychiatric Association 2000). When the two psychiatrists did not agree (in two cases), a third reviewer was consulted to help reach a final diagnosis.

Normal control subjects were defined as those individuals with no history of significant psychological problems or psychological care, psychiatric admissions, or drug detoxification and with no known history of psychiatric symptoms or substance abuse, as determined by both telephone screening and medical examiner documentation. They also had no positive toxicology results (see "Toxicology Screening" paragraph, this section). Thus, any positive history of a psychiatric problem or alcohol and/or drug abuse led to the exclusion of cases from the normal control group. Whenever possible, records from hospital admissions for medical treatment were obtained for control subjects.

Neuroleptic Medication. All neuroleptic medications recorded in available medical records of subjects with schizophrenia were converted to chlorpromazine (CPZ) equivalent doses in milligrams, as previously described (Centorrino et al 2002; Lehman and Steinwachs 1998; Woods 2003). For each subject for whom data were available, the CPZ equivalent was calculated for the following: last recorded neuroleptic dose before death (last CPZ), median daily neuroleptic dose for all neuroleptic medications prescribed throughout a subject's available treatment history (daily CPZ), and an estimate of lifetime neuroleptic exposure with the median dose multiplied by the duration of treatment in years (estimated from the earliest age of onset or age at first hospital stay) (lifetime CPZ).

Smoking. The history of cigarette smoking at the time of death was collected during the initial telephone screening. When information was missing or discrepant, habitual smoking was verified through toxicological analysis of nicotine and cotinine levels in blood or brain tissue.

Toxicology Screening. Toxicological analysis of blood, vitreous humor fluid, occipital pole, and/or urine was conducted by a forensic toxicologist for the majority of cases (Armed Forces Institute of Pathology, Office of the Armed Forces Medical Examiner, Washington DC; American Medical Laboratories, Chantilly, Virginia; or the Commonwealth of Virginia, Department of Criminal Justice Services, Divisions of Forensic Science, Northern Laboratory, Fairfax, Virginia) with gas chromatography/mass spectrometry (GC/MS) and confirmed by radioimmunoassay and confirmed with gas chromatography/mass spectrometry (GC/MS). For those subjects with missing toxicology data, frozen blood or cerebellar tissue was sent from our laboratory to the National Medical Services Laboratory to be screened by GC/MS and confirmed by enzyme-linked immunosorbant assay (ELISA) (NMS, Willow Grove, Pennsylvania). Positive toxicology was an exclusion criterion for control subjects but not for patients with schizophrenia in this study. Of the 53 subjects with schizophrenia, 37.7% of subjects ($n = 20$) had positive medical examiner toxicology screenings (2 subjects with ethanol below levels of legal intoxication, 4 subjects with resuscitative drugs, 2 with over-the-counter medication, 9 with prescribed psychotropic medication, and 3 subjects with "mixed" toxicology of prescription, [i.e., over-the-counter and resuscitative drugs]). No toxicology testing was conducted to screen for therapeutic levels of neuroleptic and antidepressant medications.

Neuropathology. Neuropathological examination was performed in all cases by a board-certified neuropathologist. Brain sections through several cortical regions and the cerebellar vermis were examined microscopically, including the use of Bielschowsky's silver stain. Cases with cerebrovascular disease (infarcts or hemorrhages), subdural hematoma, neuritic pathology, or other significant pathological features were excluded from further study. Cases with acute subarachnoid hemorrhages that were directly related to the immediate cause of death were not excluded.

Manner of Death. The cause and manner of death and any contributory causes or significant medical conditions related to death were obtained from medical examiner documents. By definition, cases were not considered as part of the normal control group if the manner of death was suicide or if death was due to drug overdose or poisoning. No subjects with schizophrenia were excluded from the study on the basis of the manner of death. The manner of death was classified as: 1) natural, 2) accidental, 3) suicide, 4) homicide, and 5) cause undetermined. Of the 10 subjects who died by suicide, 3 subjects died via blunt trauma injuries, 2 died by hanging, 1 subject died by stab wounds, 1 subject died by a gunshot wound, and 3 subjects died by prescription drug overdose. None of the subjects who died of suicide survived in a hospital setting before death.

Agonal State. To assess agonal state preceding death, data were gathered regarding specific medical conditions preceding death (e.g., coma, hypoxia, seizures) and the duration of the terminal phase. Cases were classified into four categories as previously described (Hardy et al 1985; Tomita et al 2004): 1) violent fast death (e.g., shooting or blunt trauma) with a terminal phase of < 10 min; 2) fast death of natural causes (e.g., myocardial infarction or sudden unexpected death of a person who was otherwise believed to be healthy) with a terminal phase estimated at < 1 hour; 3) intermediate death, often characterized by dying in a hospital with a terminal phase of 1–24 hours; and 4) slow death (e.g., due to cancer, bronchopneumonia, or other deaths after a long illness) with a prolonged terminal phase of more than 1 day. We subsequently decided to combine categories 2–4 into one category of prolonged agonal state (because there were no differences in pH, postmortem interval [PMI], RNA integrity number [RIN], or gene expression levels between categories 2, 3, and 4, and a group with an agonal state 1 was significantly different on these measures from the three other groups, $p < .05$).

Age. Age at death was verified by obtaining both date of birth and date of death through psychiatric records, medical examiner documents, and family interviews.

PMI. Postmortem interval was defined as the time elapsed, in hours, between the pronounced time of death and time of tissue freezing. When exact time of death was not pronounced by hospital or emergency medical personnel, PMI was estimated as the midpoint between when the individual was last seen alive and time the deceased was found. If rigor mortis was present at

time of discovery (in four cases), 4 hours (for partial rigor) or 8 hours (for full rigor) were added to this midpoint PMI estimation.

Tissue Retrieval and Processing

Brains were removed from the skull, wrapped in plastic, and transported on wet ice. The brains were hemisected, cut into 1.5 cm coronal slabs, rapidly frozen in a pre-chilled dry-ice/isopentane slurry bath (−40°C), and stored at −80°C. The time from when the tissue was stored at −80°C until the RNA was extracted was considered the freezer time (mean ± SD: 43.8 ± 2.8 months). A block of lateral superior cerebellar hemisphere was cut transversely to the folia. A portion of cerebellum was pulverized for pH measurement, as previously described (Romanczyk et al 2002). The hippocampus (83% left hemisphere) was identified on the frozen coronal slabs from the medial temporal lobe and dissected from one hemisphere with a dental drill (Cat# UP500-UG33, Brasseler, Savannah, Georgia). The lateral ventricle and the fimbria fornix were used as the mediodorsal boundary and the subiculum and underlying white matter as the ventral boundary. The adjacent parahippocampal cortex was not included in the dissection. Tissue from 2–3 slabs was pooled before pulverizing and weighing (average weight 1472 ± 572 mg). For the dorsolateral prefrontal cortex (DLPFC) dissections (average weight 1449 ± 383 mg, 83% left hemisphere), grey matter tissue from the crown of the middle frontal gyrus was obtained from the coronal slab corresponding to the middle one-third immediately anterior to the genu of the corpus callosum. Subcortical white matter was carefully trimmed from the area immediately below the middle frontal gyrus.

RNA Extraction and Quality Assessment

Tissue from DLPFC and hippocampus was pulverized and stored at −80°C. Total RNA was extracted from 300 mg of tissue with TRIZOL Reagent (Life Technologies, Grand Island, New York). The RNA from the DLPFC white matter was isolated with TRIZOL and purified with RNeasy spin columns (Qiagen, Valencia, California). This change in the protocol was caused by an overall low quality of the DLPFC white matter RNA. We wanted to ensure that systematic differences in the grey matter and white matter RNA quality would not bias the complementary DNA (cDNA) synthesis and subsequent quantitative polymerase chain reaction (RT-PCR) analysis of the white matter transcripts. The yield of total RNA was determined by spectrophotometry by measuring absorbance at 260 nm. Average yield for the hippocampus, .061% ± .014%, did not significantly differ from the yield for the DLPFC grey matter, .056% ± .023% ($p > .05$), or between the two diagnostic groups. Yield was not available for the white matter, owing to loss of data on the tissue weight (approximately 300 mg). The RNA quality was assessed with high-resolution capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California). Approximately 700 ng RNA was applied to an RNA 6000 Nano LabChip without prior heating. An RNA integrity number, obtained from the entire Agilent electrophoretic trace with the RIN software algorithm, was used for the assessment of RNA quality (scale 1–10, with 1 being the lowest and 10 being the highest RNA quality; see http://www.chem.agilent.com/temp/rad5DA20/00047692.pdf. for details). Total RNA (4 μg) was used in 50 μL of reverse transcriptase reaction to synthesize cDNA, by using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California), according to the manufacturer's protocol.

Quantitative RT-PCR

Expression levels of mRNAs were measured by quantitative real-time RT-PCR, with specific combinations of primers and probes (cat # of ABI Assays-on-Demand for β-actin [Hs99999903], porphobilinogen deaminase [PBGD] [Hs00609297], β-2-microglobulin [B2M] [Hs99999907] and β-glucuronidase [GUSB] [Hs99999908], TATA-binding protein TBP [Hs00427620]) and an ABI Prism 7900 sequence detection system with 384-well format (Applied Biosystems, Foster City, California). Each 10–20-μL reaction contained 900 nmol/L of primer, 250 nmol/L of probe, and Taqman Universal PCR Mastermix (Applied Biosystems) containing Hot Goldstar DNA Polymerase, deoxynucleoside triphosphates (dNTP)s with deoxyuridine triphosphate (dUTP), uracil-N-glycosylase, passive reference, and cDNA template generated from 100–200 ng of RNA. The PCR cycle parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 59°C or 60°C for 1 min. The PCR data were acquired from the Sequence Detector Software (SDS version 2.0, Applied Biosystems) and quantified by a standard curve method with serial dilutions of pooled cDNA derived from RNA obtained from hippocampi or DLPFC of 10–12 normal control subjects. In each experiment the R^2 value of the curve was more than .99, the slope was between −3.2 and −3.5 (amplification efficiency 96%–101%), and control subjects comprising no-template cDNA resulted in no detectable signal. The SDS software plotted real-time fluorescence intensity and selected the threshold within the exponential phase of the amplicon profiles. The software plotted a standard curve of the cycles at threshold (Ct) versus quantity of RNA. All samples were measured in a single plate for each gene, and their Ct values were in the linear range of the standard curve. All measurements were performed in triplicates and the gene expression levels calculated as an average of triplicates.

Statistical Analysis

Statistical analyses were conducted with Statistica (version 7.1., 2005, data analysis software system; StatSoft, Tulsa, Oklahoma; http://www.statsoft.com). Chi-square (χ^2) statistic was used to evaluate frequency distribution, and Student t test was used to test for differences in continuous variables between the diagnostic groups. The generalized linear model and multiple regression analyses were used to assess the contribution of independent factors (such as gender or diagnosis) and continuous predictor variables (such as pH, age) to the gene expression levels or RNA quality. Fisher least significant difference post hoc comparisons were used to evaluate group differences. Power analysis was used to calculate the numbers of subjects/group needed to achieve significant results ($p < .05$).

Results

Subject Selection

Fifty-three subjects with a documented history of schizophrenia and 90 control subjects were identified as suitable for further studies after excluding individuals having: 1) a primary psychiatric diagnosis other than schizophrenia, such as a mood disorder, substance abuse, or anxiety disorder; 2) clinical history and/or neuropathological abnormalities consistent with a primary neurological disorder such as Alzheimer's disease, Parkinson's disease, or diffuse Lewy Body disease; 3) a positive toxicology result in "control" subjects. These subjects are described in detail with respect to demographic variables in Table 1.

The brain tissue samples from these 143 subjects were used for the assessment of mRNA expression of reference genes in the

Table 1. Demographic and Clinical Summary of Cases ($n = 143$)

	Schizophrenia ($n = 53$)	Normal Control Subjects ($n = 90$)
Gender	Male – 58.5% ($n = 31$) Female – 41.5% ($n = 22$)	Male – 67.8% ($n = 61$) Female – 32.2% ($n = 29$)
Race	Caucasian – 43.4% ($n = 23$) African-American – 50.9% ($n = 27$) Hispanic – 5.7% ($n = 3$)	Caucasian – 32.2% ($n = 29$) African-American – 60.0% ($n = 54$) Hispanic – 4.5% ($n = 4$) Asian – 3.3% ($n = 3$)
Age At death	50.6 ± 17.6 years	41.7 ± 14.8 years
pH	6.42 ± .32	6.55 ± .33
Postmortem Interval	39.7 ± 19.5 hours	32.0 ± 14.3 hours
Manner Of Death	Natural – 67.9% ($n = 36$) Accidental – 11.3% ($n = 6$) Suicide – 18.9% ($n = 10$) Undetermined – 1.9% ($n = 1$)	Natural – 74.4% ($n = 67$) Accidental – 8.9% ($n = 8$) Homicide – 16.7% ($n = 15$)
Agonal Duration Rating	1: 18.9% ($n = 10$) 2, 3, or 4: 79.2% ($n = 42$) Unknown: 1.9% ($n = 1$)	1: 21.1% ($n = 19$) 2, 3, or 4: 78.9% ($n = 71$)
Smoking Status At Time Of Death	Habitual Smoker – 67.9% ($n = 36$) Non-Smoker – 32.1% ($n = 17$)	Habitual Smoker – 26.7% ($n = 24$) Non-Smoker – 73.3% ($n = 66$)
Schizophrenia Subtype	CUS – 37.8% ($n = 20$) CDS – 35.8% ($n = 19$) CPS – 9.4% ($n = 5$) CCS – 3.8% ($n = 2$) Schizoaffective – 13.2% ($n = 7$)	N/A
Age Of Onset Of Illness	23.4 ± 9.6 years	N/A
Duration Of Illness	27.7 ± 16.1 years	N/A
Chlorpromazine Equivalents[a]	Last Dose – 612 ± 534 mg qd Average Daily Dose – 510 ± 297 mg qd Lifetime Dose – 4,401,830 ± 4,406,379 mg	N/A

Agonal duration rating of 1: < 10 min; 2: 10 min to 1 hour; 3: 1 hour to 24 hours; 4: > 24 hours. CUS, chronic undifferentiated schizophrenia; CDS, chronic disorganized schizophrenia; CPS, chronic paranoid schizophrenia; CCS, chronic catatonic schizophrenia; Schizoaffective, Schizoaffective Disorder.

[a]Available for $n = 47$.

hippocampus and DLPFC grey and white matter by quantitative RT-PCR.

Confounding Variables in Patients With Schizophrenia Versus Control Subjects

We first examined whether patients with schizophrenia ($n = 53$) differed from normal control subjects ($n = 90$) in demographic and tissue-related measures. The data indicated that patients with schizophrenia more frequently were smokers [$\chi^2(1) = 22.8$, $p < .0001$, 67.9% vs. 25.6%] and were older at death and their brain samples had lower pH, longer PMI, and lower quality of total RNA in all three brain regions sampled (hippocampus and DLPFC grey and white matter); all p values < .05 (t tests for continuous variables; Tables 1 and 2). Not surprisingly, because by design no subjects who committed suicide were included in the control group, patients with schizophrenia also differed from control subjects in the manner of death [$\chi^2(3) = 31.3$, $p < .001$].

Variables Contributing to Gene Expression Levels

Next, we measured the expression of a panel of reference or so called "housekeeping" genes expected, in principle, to show a relatively stable expression independent of age-related changes, treatment, or disease state and frequently used for normalization in quantitative PCR experiments (Bustin et al 2005; Huggett et al 2005). We chose PBGD, TATA-binding protein (TBP), β-glucuronidase (GUSB), β-actin, and β-2-microglobulin (B2M), because they show low to moderate expression levels in the human brain tissue (i.e., match the expression levels of a majority of target genes of interest) and are relatively stable across individuals and tissues (Lupberger et al 2002; Tricarico et al 2002). We also calculated a normalizing factor (NF; a geometric mean of expression levels, $NF = [g_1 \times g_2 \times g_3 \times g_4]^{1/4}$, where g_{1-4} are the expression levels of four individual genes) (i.e., a weighted expression of these transcripts, considered to be more appropriate for normalization in relative RT-PCR quantification studies of target genes than individual transcripts [Vandesompele et al 2002]). The same four genes (PBGD, B2M, β-actin, and GUSB) were used for calculating NF in every brain region.

There was considerable inter-individual variability in the expression of reference genes in all of the brain regions examined (Table 2). A coefficient of variation (% CV) for the measured transcripts ranged from 40% to 84% in the hippocampus, 43% to 52% in the DLPFC grey matter, and 64% to 80% in the DLPFC white matter, depending on the transcript. Although variability in the levels of transcription appeared slightly higher in patients with schizophrenia than in control subjects, the differences were unremarkable (Table 2). We also found that there was considerable variability in RIN values between regions (Table 2). The difference between DLPFC grey and white is most likely caused by different extraction details. The difference between hippocampus and DLPFC is harder to explain but might be related to differences in sensitivity to RNA degradation between the two regions. However, it is also worth noting that despite relatively high RIN values and lowest variability in RNA quality in the DLPFC white matter as compared with the hippocampus and DLPFC grey matter, the variability in gene expression levels in

Table 2. Differences Between Diagnostic Groups (expressed as %Control Subject) and Inter-Individual Variability (expressed as a coefficient of variation, %CV).

Variable	%Control Subject	*p*	Control Subject %CV	Schizophrenic %CV
Age	121.4[a]	.001	35.6	34.8
pH	98.1[a]	.03	5.1	5.0
PMI	123.9[a]	.009	44.7	49.2
RIN_HIP	89.7[a]	.04	28.2	32.2
RIN_PFC	88.6[a]	.05	31.1	42.1
RIN_WM	86.1[a]	.0003	16.5	26.6
Hippocampus				
Actin	96.8	>.5	86.0	79.9
TBP	91.8	>.5	58.7	83.6
B2M	84.7	>.2	65.4	73.8
PBGD	69.8[a]	.009	67.5	82.7
GUSB	91.7	>.2	40.8	51.0
NF	99.3	>.5	42.3	40.0
Gray Matter_DLPFC				
Actin	81.7[a]	.04	43.7	52.2
B2M	88.2	>.2	54.9	43.0
PBGD	83.2[a]	.056	42.9	49.5
GUSB	108.9	>.5	56.3	44.8
NF	106.2	>.5	50.2	46.6
White Matter_DLPFC				
Actin	60.5[a]	.004	78.0	79.9
B2M	63.0[a]	.01	89.4	70.5
PBGD	78.3[a]	.05	61.5	64.1
GUSB	81.2	>.1	57.3	73.3
NF	71.5[a]	.01	64.8	66.9

PMI, postmortem interval; RIN, RNA integrity number; HIP, hippocampus; DLPFC, dorsolateral prefrontal cortex; WM, white matter; TBP, TATA-binding protein; B2M, β-2-microglobulin; PBGD, porphobilinogen transaminase; GUSB, β-glucuronidase; NF, normalizing factor.

[a] $p < .05$.

the white matter was at least as high as in other tissues (Table 2). We found weak correlations between the regions: RIN in the hippocampal samples significantly correlated with RIN of the DLPFC white and grey matter (both $r = .2$, $p < .05$), but there was no significant correlation between DLPFC white and grey matter RINs ($r = .13$, $p > .05$).

Multiple regression analysis in the entire sample that included both patients with schizophrenia and control subjects revealed that the expression of housekeeping genes was significantly affected by the confounding factors entered in the linear model. Their contribution to the variance varied from 6% to 50% (adjusted R^2 .06–0.50, p values $< .05$), depending on the transcript species and the brain region in which it was measured (Table 3). Race was not entered in the model, because neither expression levels of these control genes nor demographic- or tissue-related measures differed between the races. Also, the numbers of Asian and Hispanic subjects were small ($n = 3$ and $n = 7$, respectively). Gender had no effect on the expression of any housekeeping genes. Among the housekeeping genes examined, hippocampal PBGD mRNA (a relatively rare transcript) was the most sensitive to the variables considered in the linear model, whereas the GUSB transcript in the hippocampus and DLPFC grey matter was the least sensitive and its expression was not significantly affected by the variables entered (although it was still highly variable between individuals). In the DLPFC white matter, all transcripts showed high inter-individual variability and were highly affected by confounding variables (Tables 2 and 3). In summary, there were marked regional and transcript-specific differences in the variability between subjects and susceptibility to confounding factors.

For a majority of mRNAs, the best predictor of individual gene expression was total RNA quality (RIN), regardless of the brain tissue examined (i.e., higher RIN scores predicted higher levels of expression of TBP, B2M, and PBGD in the hippocampus, β-actin, and B2M in the DLPFC grey matter and all measured transcripts in the DLPFC white matter as well as significantly higher normalizing factors in all brain tissues) (Table 3). Other important unique contributors to the expression levels of housekeeping genes in these tissues were PMI, pH, and agonal state. Short PMI and high pH values predicted higher gene expression in the hippocampus, and agonal state was a strong predictor of the transcript level in the DLPFC white matter (i.e., longer agonal state predicted lower expression of housekeeping genes). Age contributed less to the gene expression prediction (i.e., only PBGD in the hippocampus and the grey matter of the DLPFC and β-actin in the grey matter DLPFC showed significant negative correlations with age). It should also be noted that age and PMI predicted NF values in the hippocampus and agonal state predicted NF values in the DLPFC white matter.

Because overall RNA quality (RIN) seemed to be a major factor in gene expression levels in the brain regions studied, we further interrogated which of the examined factors best determined RIN. We found that hippocampal RIN was best predicted by pH, which accounted for 23% variance [$F = 15.5$, adj $R^2 = .23$, $p < .00001$, $\beta = .47$]. The DLPFC grey matter RNA quality was best predicted by pH and PMI, together accounting for 15% variance [$F = 7.1$, adj. $R^2 = .15$, $p < .0001$, β values .36 and −.20, respectively], indicating that high pH values and low PMI were associated with better RNA quality. The RNA quality of the white matter DLPFC was best predicted by both pH [$\beta = .24$, $p = .003$] and smoking [$\beta = -.25$, $p = .002$], together contributing 13% variance [$F = 7.8$, $p < .0001$] and indicating that high pH values and no smoking history predicted better RNA quality. Interestingly, brain tissue of smokers had significantly lower pH than nonsmokers [6.44 vs. 6.55, respectively, $t(141) = 1.98$, $p < .05$].

Power Analysis

With power analysis, we explored whether this sample size would be sufficient to detect group differences in gene expression of 1.25-fold to 2-fold. For this, we estimated sample sizes needed to reject a null hypothesis of no difference between the diagnostic groups in the expression of housekeeping genes examined in this study. The data used in the power calculations were means and standard deviations of housekeeping genes in patients with schizophrenia and control subjects.

The results indicated that for a 95% confidence level and 95% statistical power, sample sizes ranging from 60 to 232/group would be needed to allow detection of 1.25-fold differences, depending on inter-individual variability (Table 4). Genes with low inter-individual variability (e.g., GUSB in the hippocampus and PBGD in the DLPFC grey) would require approximately 15 to 60 subjects/group to detect 1.5-fold and 1.25-fold differences, respectively, whereas genes with high expression variability would require approximately 50 to 200 subjects/group to detect 1.5-fold and 1.25-fold differences (Table 4). Relaxing the statistical power requirement from 95% to 80% (i.e., somewhat compromising our ability to detect an effect, given that the effect actually exists)

Table 3. Multiple Regression Analysis in the Whole Cohort (schizophrenic and control subjects)

		Dx	Age	Gender	RIN	pH	PMI	Sm	MD	AS
Hippocampus										
	adj R^2	β	β	β	β	β	β	β	β	β
Actin	.11[a]	ns	ns	ns	ns	.24[b]	−.24[b]	ns	.28[c]	ns
TBP	.33[d]	ns	ns	ns	.49[d]	ns	−.18[c]	ns	ns	ns
B2M	.23[d]	ns	ns	ns	.46[d]	ns	−.18[c]	ns	ns	ns
PBGD	.50[d]	ns	−.16[c]	ns	.44[d]	.19[b]	−.18[b]	ns	ns	ns
GUSB	.02	ns	ns	ns	ns	ns	ns	ns	ns	ns
NF	.12[a]	ns	−.20[c]	ns	.19[c]	ns	−.21[c]	ns	ns	ns
DLPFC-Grey										
Actin	.14[a]	ns	−.26[b]	ns	.17[c]	ns	ns	ns	ns	ns
B2M	.06[c]	ns	ns	ns	.22[c]	ns	ns	ns	ns	ns
PBGD	.012[b]	ns	−.21[b]	ns	ns	ns	ns	ns	ns	ns
GUSB	.01	ns	ns	ns	ns	ns	ns	ns	ns	ns
NF	.08[c]	ns	ns	ns	.24[c]	ns	ns	ns	ns	ns
DLPFC-White										
Actin	.29[d]	ns	ns	ns	.45[d]	ns	ns	ns	ns	−.24[a]
B2M	.20[d]	ns	ns	ns	.44[d]	ns	ns	ns	ns	ns
PBGD	.23[d]	ns	ns	ns	.44[d]	ns	ns	ns	ns	−.19[b]
GUSB	.25[d]	ns	ns	ns	.47[d]	ns	ns	ns	ns	−.21[b]
NF	.28[d]	ns	ns	ns	.50[d]	ns	ns	ns	ns	−.19[b]

adj R^2, adjusted coefficient of determination; β, standardized regression coefficient; Dx, diagnosis (1-control subjects, 2-schizophrenic); Gender, 1-female, 2-male; Sm, smoking history (1-No, 2-Yes); MD, manner of death; AS, agonal state (1: < 10 min; 2: > 1 hr); DLPFC, dorsolateral prefrontal cortex; other abbreviations as in Table 2.

[a] $p < .001$.
[b] $p < .01$.
[c] $p < .05$.
[d] $p < .0001$.

would allow detection of at least 1.25-fold differences for most genes with this cohort.

Discussion

In this study, we report the results of a series of analyses aimed at parsing variance in gene expression in postmortem brain tissue. We also estimated a fold of between-group differences in gene expression that could be resolved with these brain tissue samples. We confirmed the often-reported observations that RNA quality, pH, PMI, and age result in changes in gene expression and that patients with schizophrenia as a sample population tend to have more extreme values in these confounders than the control specimens. Patients with schizophrenia were significantly older at the time of death than control subjects, and their brain tissue samples had

Table 4. Calculations of Sample Sizes Sufficient to Detect 1.25-, 1.5-, and 2-Fold Differences in Gene Expression at 95% Confidence Level and 95% and 80% Statistical Power

	Power 95%			Power 80%		
	1.25-Fold	1.5-Fold	2-Fold	1.25-Fold	1.5-Fold	2-Fold
Hippocampus (*n*)						
Actin	232	58	15	133	33	8
TBP	163	43	11	84	21	5
B2M	113	30	8	69	17	4
PBGD	84	21	5	48	12	3
GUSB	60	15	4	35	9	2
GrayMatter_DLPFC						
Actin	70	17	4	40	10	2
B2M	75	19	5	43	11	3
PBGD	64	16	4	38	9	2
GUSB	88	22	5	37	10	3
WhiteMatter_DLPFC						
Actin	137	34	9	78	20	5
B2M	145	40	10	80	22	6
PBGD	106	27	6	63	16	4
GUSB	119	30	8	68	17	4

PMI, postmortem interval; RIN, RNA integrity number; HIP, hippocampus; DLPFC, dorsolateral prefrontal cortex; WM, white matter; TBP, TATA-binding protein; B2M, β-2-microglobulin; PBGD, porphobilinogen transaminase; GUSB, β-glucuronidase; NF, normalizing factor.

lower pH, longer PMI, and lower quality total RNA than those from normal control subjects.

Patients with schizophrenia also smoked at much higher rates. Smoking, age, and manner of death were the best predictors of a diagnostic group status. This is no surprise, because it is well known that patients with schizophrenia smoke more frequently than the general population (Dalack et al 1998; Hughes et al 1986; Leonard et al 2001). However, nicotine as a psychoactive substance might alter a variety of neurochemical parameters in the brain, and its potential impact on gene expression cannot be discounted. Moreover, we found that smoking was a unique contributing factor to RNA quality in the white matter DLPFC and also to pH. Although the reason for this is not entirely clear, there is ample evidence to suggest that white matter might be particularly vulnerable to injury, including damage caused by smoking and oxidative stress as well as antemortem hypoxia, the deleterious biological effects of cigarette smoke (Back and Rivkees 2004; Ho et al 2005; Hoshino et al 2005; Park et al 2005). Importantly, reactive oxygen species and other free radicals generated by cigarette smoke can induce RNA damage, affect in vitro transcript quantification by blocking reverse transcriptase, and affect PCR amplification of mRNA (Martinet et al 2004). Smoking and nicotine administration also increase the risk for cerebrovascular disease, specifically microvascular disease, even in the absence of neuropathological evidence of overt infarction and thus might cause cell damage and changes in RNA integrity (Hawkins et al 2002). In conclusion, our data suggest that smoking seems to be an important factor contributing to total RNA quality and subsequently mRNA expression levels.

Previous reports indicate that the pH in brains of patients with schizophrenia was lower and PMI longer than in control subjects (Eastwood and Harrison 2005; Prabakaran et al 2004; Torrey et al 2005). Prabakaran et al (2004) suggested that decreased pH in the prefrontal cortex of patients with schizophrenia was not a pre- or postmortem artifact but rather was related to the underlying disease process, perhaps metabolic abnormalities, mitochondrial dysfunction, and oxidative stress. It is probable that smoking can again be a culprit in the finding of lower brain pH associated with schizophrenia, because oxidative stress, a recognized effect of smoking, impairs regulation in pH homeostasis and increases acidosis in rat brain (Sipos et al 2005). We found that brain tissue of smokers had significantly lower pH than nonsmokers, independent of disease status. It is also possible that lower pH might be due to other causes, such as the elevation of arterial carbon dioxide by direct inhalation of smoke or chronic pulmonary disease.

The difference in age at the time of death between patients with schizophrenia and normal control subjects reflects the typical ascertainment bias in the nature of individuals that are autopsied by a medical examiner's office (i.e., more often young control males dying from homicide or accidents). Age is believed to contribute to pH by slower modes of death in elderly (Harrison et al 1995). Age was, however, identified as a unique discriminator between groups, independent of manner of death, and a unique contributor to the expression of some genes (PBGD and β-actin), where manner of death did not play a significant role. The reason for this might be that age at death is a much more accurate and sensitive measure than a rather imprecise, descriptive, and sometimes subjective manner of death. Although agonal state scored significantly as a factor in gene expression in our study as well as in other studies (Hardy et al 1985; Tomita et al 2004), agonal state is difficult to determine accurately, particularly in subjects autopsied in medical examiners' offices. Both negative and positive results regarding the influence of agonal state should, perhaps, be taken with caution.

Not surprisingly, we found that the most important factor affecting expression levels of housekeeping genes, regardless of the diagnosis or brain tissue type, was total RNA quality. Evaluation of RNA integrity is an essential step to improve the reliability and accuracy of gene expression data obtained with modern amplification techniques, such as real-time quantitative RT-PCR or microarrays (Miller et al 2004). An index of total RIN provided by the Agilent Bioanalyzer 2001 software seems to be a useful measure of RNA quality. In our and other investigators' experience, RNA analyses with the Agilent RNA Nano Assay represent a marked improvement over 28S:18S ratio or compared with those performed on ethidium bromide-stained agarose gel electrophoresis (BKL, unpublished observations; Miller et al 2004; Ricicova and Palkova 2003).

Our results show that total RNA quality is the main potential confounding factor in gene expression studies with the postmortem human brain tissue and that, in agreement with numerous previous reports (see introductory section), pH, PMI, age, agonal state, and smoking might also play roles independent of their effects on RNA quality (Harrison 1999; Harrison and Kleinman 2000). We also have shown that the impact of these variables is gradual, with no obvious cut-off points, and highly dependent on the transcript and brain region analyzed. Proper matching of diagnostic cohorts requires controlling for these factors. Covariance for these factors is another strategy for adjusting for differences in these confounders, but it is potentially problematic because some of these relationships might not be strictly linear. However, without deeper understanding of the underlying causal mechanisms of particular mRNA changes, drawing universal guidelines or recommendations about the exclusion or

Table 5. Selected Cohorts

	n	Race	Gender	Age	PMI	pH	RIN
Hippocampus							
Control Subjects	73	44AA/2A/3H/24C	19F/54M	41.6 ± 14.8	31.4 ± 14.4	6.61 ± .26	5.7 ± 1.0
Schizophrenic	32	17AA/1H/14C	9F/23M	46.3 ± 15.2	35.2 ± 15.2	6.53 ± .27	5.4 ± 1.1
DLPFC-Grey							
Control Subjects	70	41AA/3A/4H/22C	19F/51M	41.7 ± 15.2	32.6 ± 14.1	6.61 ± .24	6.7 ± 1.3
Schizophrenic	36	17AA/3H/16C	15F/21M	49.8 ± 18.3	36.1 ± 16.8	6.51 ± .27	6.5 ± 1.6
DLPFC-White							
Control Subjects	73	45AA/2A/3H/23C	25F/48M	41.6 ± 16.3	32.2 ± 14.3	6.60 ± .28	6.7 ± 1.0
Schizophrenic	30	20AA/3H/7C	12F/18M	48.7 ± 16.0	39.0 ± 19.7	6.53 ± .24	6.3 ± 1.1

DLPFC, dorsolateral prefrontal cortex; AA, African American; A, Asian; H, Hispanic; C, Caucasian; PMI, postmortem interval; RIN, RNA integrity number.

inclusion criteria is not possible at this time. Thus, we suggest selecting optimized cohorts for studying gene expression by using empirically derived criteria that might vary depending on the transcript, brain region, and so forth and including multiple variables in the analysis. Of course, the numbers of subjects must be sufficiently large to conduct such analysis (i.e., $n > 10$ / predicting variable). For instance, in our cohort, excluding samples with RNA quality below RIN 4.0 (3.8 for the hippocampus), pH < 6.0, age at death > 80 years, and PMI > 72 hours (Table 5) resulted in groups of patients with schizophrenia and control subjects that did not significantly differ in RIN or pH but still significantly differed in age and PMI ($p < .05$). Importantly, in these selected cohorts, there were no significant differences between patients with schizophrenia and control subjects in the levels of expression of any of the housekeeping genes in each brain region examined (all $p > .1$). The variance in the expression levels across transcripts, regions, and diagnostic groups did not diminish, however, in any appreciable way (CV% between 40% and 80%).

Normalization of the target gene expression to the individual housekeeping gene expression or to the normalizing factors will considerably diminish the inter-individual variability of target genes, thereby increasing our ability to detect relatively subtle differences of 1.25-fold or less. Reducing variability might be particularly important for the studies of the effects of genotype on gene expression, where genotypic groups are often small and dependent on the frequency of a particular polymorphism. For instance, for the rare allelic frequencies of 10%, the genotypic groups in our cohorts will be as small as 3–7 subjects / rare allele / diagnostic group. Thus, under these circumstances, only differences as large as 2-fold might be detectable (Table 4).

Summary

We have identified and/or validated factors that should be considered when assembling a cohort of postmortem brains for the investigation of gene expression. We found that the most important factor affecting expression levels of housekeeping genes, regardless of the diagnosis or brain tissue type, was total RNA quality. Accurate and reliable assessment of RNA quality should thus be an essential step in gene expression studies. Other factors to be considered include pH, PMI, age, agonal state, and smoking history, but they are transcript- and tissue-specific. Therefore, proper matching of diagnostic cohorts requires controlling for these factors. We suggest that each data set derived from an experimental cohort be examined individually rather than imposing arbitrary cut-offs or exclusion criteria and that predicting variables be included in the analysis of the expression data.

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American Psychiatric Association (2000): *Diagnostic and Statistical Manual of Mental Disorders, 4th ed., Text Revision*. Washington DC: American Psychiatric Association.

Back SA, Rivkees SA (2004): Emerging concepts in periventricular white matter injury. *Semin Perinatol* 28:405–414.

Bahn S, Augood SJ, Ryan M, Standaert DG, Starkey M, Emson PC (2001): Gene expression profiling in the post-mortem human brain—no cause for dismay. *J Chem Neuroanat* 22:79–94.

Barton AJ, Pearson RC, Najlerahim A, Harrison PJ (1993): Pre- and postmortem influences on brain RNA. *J Neurochem* 61:1–11.

Bray NJ, Preece A, Williams NM, Moskvina V, Buckland PR, Owen MJ, O'Donovan MC (2005): Haplotypes at the dystrobrevin binding protein 1 (DTNBP1) gene locus mediate risk for schizophrenia through reduced DTNBP1 expression. *Hum Mol Genet* 14:1947–1954.

Burke WJ, O'Malley KL, Chung HD, Harmon SK, Miller JP, Berg L (1991): Effect of pre- and postmortem variables on specific mRNA levels in human brain. *Brain Res Mol Brain Res* 11:37–41.

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005): Quantitative real-time RT-PCR—a perspective. *J Mol Endocrinol* 34:597–601.

Centorrino F, Eakin M, Bahk WM, Kelleher JP, Goren J, Salvatore P, et al (2002): Inpatient antipsychotic drug use in 1998, 1993, and 1989. *Am J Psychiatry* 159:1932–1935.

Craddock N, O'Donnovan MC, Owen MJ (2005): The genetics of schizophrenia and bipolar disorder: Dissecting psychosis. *J Med Genet* 42: 193–204.

Dalack GW, Healy DJ, Meador-Woodruff JH (1998): Nicotine dependence in schizophrenia: Clinical phenomena and laboratory findings. *Am J Psychiatry* 155:1490–501.

Deep-Soboslay A, Akil M, Martin CE, Bigelow LB, Herman MM, Hyde TM, Kleinman JE (2005): Reliability of psychiatric diagnosis in postmortem research. *Biol Psychiatry* 57:96–101.

Eastwood SL, Harrison PJ (2005): Decreased expression of vesicular glutamate transporter 1 and complexin II mRNAs in schizophrenia: Further evidence for a synaptic pathology affecting glutamate neurons. *Schizophr Res* 73:159–172.

Egan MF, Straub RE, Goldberg TE, Yakub I, Callicott JH, Hariri AR, et al (2004): Variation in GRM3 affects cognition, prefrontal glutamate, and risk for schizophrenia. *Proc Natl Acad Sci U S A* 101:12604–12609.

First MB, Spitzer RL, Gibbon M, Williams JBW (1997): *User's Guide for the Structured Clinical Interview for DSM-IV Axis I Disorders—Clinician Version (SCID-CV)*. Washington DC: American Psychiatric Press.

Hardy JA, Wester P, Winblad B, Gezelius C, Bring G, Eriksson A (1985): The patients dying after long terminal phase have acidotic brains; implications for biochemical measurements on autopsy tissue. *J Neural Transm* 61:253–264.

Harrison PJ (1996): Advances in post mortem molecular neurochemistry and neuropathology: Examples from schizophrenia research. *Br Med Bull* 52: 527–38.

Harrison PJ (1999): The neuropathology of schizophrenia. A critical review of the data and their interpretation. *Brain* 122:593–624.

Harrison PJ, Heath PR, Eastwood SL, Burnet PW, McDonald B, Pearson RC (1995): The relative importance of premortem acidosis and postmortem interval for human brain gene expression studies: Selective mRNA vulnerability and comparison with their encoded proteins. *Neurosci Lett* 200:151–154.

Harrison PJ, Kleinman JE (2000): Methodological Issues. In: Harrison PJ, Roberts G, editors. *The Neuropathology of Schizophrenia*. New York: Oxford University Press, 339–350.

Harrison PJ, Weinberger DR (2005): Schizophrenia genes, gene expression, and neuropathology: On the matter of their convergence. *Mol Psychiatry* 10:40–68.

Hawkins BT, Brown RC, Davis TP (2002): Smoking and ischemic stroke: A role for nicotine? *Trends Pharmacol Sci* 23:78–82.

Ho SP, Chan-Yeung M, Chow KK, Ip MS, Mak JC (2005): Antioxidant enzyme activities in healthy Chinese adults: Influence of age, gender and smoking. *Respirology* 10:305–309.

Hoshino S, Yoshida M, Inoue K, Yano Y, Yanagita M, Mawatari H, et al (2005): Cigarette smoke extract induces endothelial cell injury via JNK pathway. *Biochem Biophys Res Commun* 329:58–63.

Huggett J, Dheda K, Bustin S, Zumla A (2005): Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* 6:279–284.

Hughes JR, Hatsukami DK, Mitchell JE, Dahlgren LA (1986): Prevalence of smoking among psychiatric outpatients. *Am J Psychiatry* 143:993–997.

Hulette CM (2003): Brain banking in the United States. *J Neuropathol Exp Neurol* 62:715–22.

Johnston NL, Cervenak J, Shore AD, Torrey EF, Yolken RH (1997): Multivariate analysis of RNA levels from postmortem human brains as measured by three different methods of RT-PCR. Stanley Neuropathology Consortium. *J Neurosci Methods* 77:83–92.

Kelly TM, Mann JJ (1996): Validity of DSM-III-R diagnosis by psychological autopsy: A comparison with clinician ante-mortem diagnosis. *Acta Psychiatr Scand* 94:337–343.

Kingsbury AE, Foster OJ, Nisbet AP, Cairns N, Bray L, Eve DJ, et al (1995): Tissue pH as an indicator of mRNA preservation in human post-mortem brain. *Brain Res Mol Brain Res* 28:311–318.

Kleinman JE, Hyde TM, Herman MM (1995): Methodological issues in the neuropathology of mental illness. In: Bloom FE, Kupfer DJ, editors. *Psychopharmacology: The Fourth Generation of Progress*. New York: Raven Press, 859–864.

Law AJ, Lipska BK, Weickert CS, Hyde TM, Straub RE, Hashimoto R, et al (2006): Neuregulin (NRG1) transcripts are differentially expressed in schizophrenia and regulated by 5′ SNPs associated with the disease. *Proc Natl Acad Sci U S A* 103:6747–6752.

Lehman AF, Steinwachs DM (1998): Translating research into practice: The Schizophrenia Patient Outcomes Research Team (PORT) treatment recommendations. *Schizophr Bull* 24:1–10.

Leonard S, Adler LE, Benhammou K, Berger R, Breese CR, Drebing C, et al (2001): Smoking and mental illness. *Pharmacol Biochem Behav* 70:561–570.

Lewis DA (2002): The human brain revisited: Opportunities and challenges in postmortem studies of psychiatric disorders. *Neuropsychopharmacology* 26:143–154.

Lipska BK, Peters T, Halim N, Mitkus S, Shannon Weickert C, Matsumoto M, et al (2006): Expression of DISC1 binding partners is reduced in schizophrenia and associated with DISC1 SNPs. *Hum Mol Gen* 15:1245–1258.

Lupberger J, Kreuzer KA, Baskaynak G, Peters UR, le Coutre P, Schmidt CA (2002): Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. *Mol Cell Probes* 16:25–30.

Martinet W, de Meyer GR, Herman AG, Kockx MM (2004): Reactive oxygen species induce RNA damage in human atherosclerosis. *Eur J Clin Invest* 34:323–327.

Miller CL, Diglisic S, Leister F, Webster M, Yolken RH (2004): Evaluating RNA status for RT-PCR in extracts of postmortem human brain tissue. *Biotechniques* 36:628–633.

Park MK, Jo I, Park MH, Kim TK, Jo SA, Shin C (2005): Cerebral white matter lesions and hypertension status in the elderly Korean: The Ansan Study. *Arch Gerontol Geriatr* 40:265–273.

Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JT, Griffin JL, et al (2004): Mitochondrial dysfunction in schizophrenia: Evidence for compromised brain metabolism and oxidative stress. *Mol Psychiatry* 9:684–697.

Preece P, Cairns NJ (2003): Quantifying mRNA in postmortem human brain: Influence of gender, age at death, postmortem interval, brain pH, agonal state and inter-lobe mRNA variance. *Brain Res Mol Brain Res* 118:60–71.

Preece P, Virley DJ, Costandi M, Coombes R, Moss SJ, Mudge AW, et al (2003): An optimistic view for quantifying mRNA in post-mortem human brain. *Brain Res Mol Brain Res* 116:7–16.

Ravid R, Van Zwieten EJ, Swaab DF (1992): Brain banking and the human hypothalamus—factors to match for, pitfalls and potentials. *Prog Brain Res* 93:83–95.

Ricicova M, Palkova Z (2003): Comparative analyses of Saccharomyces cerevisiae RNAs using Agilent RNA 6000 Nano Assay and agarose gel electrophoresis. *FEMS Yeast Res* 4:119–122.

Romanczyk TB, Weickert CS, Webster MJ, Herman MM, Akil M, Kleinman JE (2002): Alterations in trkB mRNA in the human prefrontal cortex throughout the lifespan. *Eur J Neurosci* 15:269–280.

Sipos H, Torocsik B, Tretter L, Adam-Vizi V (2005): Impaired regulation of pH homeostasis by oxidative stress in rat brain capillary endothelial cells. *Cell Mol Neurobiol* 25:141–151.

Tomita H, Vawter MP, Walsh DM, Evans SJ, Choudary PV, Li J, et al (2004): Effect of agonal and postmortem factors on gene expression profile: Quality control in microarray analyses of postmortem human brain. *Biol Psychiatry* 55:346–352.

Torrey EF, Barci BM, Webster MJ, Bartko JJ, Meador-Woodruff JH, Knable MB (2005): Neurochemical markers for schizophrenia, bipolar disorder, and major depression in postmortem brains. *Biol Psychiatry* 57: 252–260.

Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, et al (2002): Quantitative real-time reverse transcription polymerase chain reaction: Normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. *Anal Biochem* 309:293–300.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:RESEARCH0034.

Woods SW (2003): Chlorpromazine equivalent doses for the newer atypical antipsychotics *J Clin Psychiatry* 64:663–667.

Xu B, Wratten N, Charych EI, Buyske S, Firestein BL, Brzustowicz LM (2005): Increased expression in dorsolateral prefrontal cortex of CAPON in schizophrenia and bipolar disorder. *PLoS Med* 2:e263.

Zalcman S, Endicott J (1983): *Diagnostic Evaluation After Death*. Developed for NIMH Neurosciences Research Branch, Department of Research Assessment and Training. New York, NY: New York State Psychiatric Institute.