Substantial advances in basic neuroscience, including understanding the molecular and cellular pathways of neural transmission and plasticity, offer new insights into the mechanisms that underlie brain dysfunction in psychiatric illnesses. However, testing of these ideas requires the examination of the human brain at the molecular, cellular, and circuitry levels, a resolution that can currently be obtained only by direct examination of brain tissue. Unlike other organ systems, brain tissue cannot be assessed via biopsy in the living patient with a psychiatric disorder; therefore, the availability of brain samples obtained at autopsy is critical for advancing the field. As a result, interest in postmortem studies has been renewed over the last two decades, despite previous criticisms that the problems and confounds were too numerous or challenging to control (Plum, 1972). Pioneering work by the leaders of the renaissance in postmortem studies of psychiatric illness (Bracha and Kleinman, 1984; Kleinman et al., 1985; Bracha and Kleinman, 1986; Kovelman and Scheibel, 1986; Benes and Bird, 1987; Benes, Majocha, et al., 1987; Benes, Matthysse, et al., 1987; Mann, Arango, et al., 1989; Mann, Marzuk, et al., 1989; Arango et al., 1990; Benes, 1998) and the application of new investigational techniques and more rigorous methods of quantification have led to the continuing improvement in the design and conduct of postmortem studies.

The critical levels of resolution provided by postmortem studies are complementary to genetic and brain imaging approaches to the study of mental illnesses. For example, for many types of genetic findings, the potential biological significance of a putative susceptibility genetic variant is strengthened by evidence that the expression level of the encoded transcription product is altered in the brain. Similarly, findings from neuroimaging studies of regional patterns of functional activation, ligand receptor binding, or volumetric differences depend upon postmortem studies to identify or confirm the neurobiological basis for the observations.

Advances in understanding the molecular, cellular, and circuitry alterations in psychiatric illnesses depend directly upon the number and quality of available brain specimens and the appropriate implementation of experimental designs that maximize the yield of interpretable data and minimize the influence of confounding factors. For example, a recent study of the methodological considerations for gene expression profiling in postmortem human brain found that clinical data, tissue, RNA, and technique quality must all be considered as potential confounding factors (Atz et al., 2007). Consequently, advances in postmortem human brain studies require (1) well-characterized brain specimens, (2) well-conceptualized or designed studies, and (3) well-controlled confounds (Lewis, 2002). In the following sections, we discuss the approaches that can facilitate the achievement of these goals.

CHARACTERIZATION OF THE SUBJECTS

Clinical Diagnosis

As with any area of investigation of psychiatric disorders, postmortem studies depend upon syndromal diagnoses based on clinical features. Thus, as in genetic neuroimaging or other studies, the information value of a postmortem study is only as good as the amount and accuracy of the knowledge that supports the presence of the disorder under investigation. Consequently, the same emphasis on the systematic acquisition of clinical data and of reliability in the diagnostic process that characterizes state-of-the-art clinical studies must be applied in postmortem investigations. In addition, it is also essential to know, with a similar level of certainty, that the normal comparison subjects do not have a history of a psychiatric disorder. For example, in our experience with subjects identified through a medical examiner’s office, subjects for whom the initially available clinical records indicated no history of psychiatric illness are commonly found to have a diagnosable psychiatric disorder, especially substance abuse or dependence.
Just as structured interviews, with documented reliability, are state-of-the-art for clinical studies, the same diagnostic rigor is an essential element in the characterization of subjects for postmortem studies. Structured, antemortem diagnostic assessments have been used quite effectively in postmortem studies of subjects who are elderly, chronically hospitalized, and with schizophrenia (Arnold et al., 1998; Sokolov et al., 2000). The course of illness and substantial cognitive impairment of many of these subjects suggest that they represent a unique and important subgroup of subjects with schizophrenia, but their distinctive clinical features limit the extent to which findings from these studies can be generalized to other forms or earlier stages of the illness.

Such prospective, antemortem assessments are not possible for studies of individuals in the early to mid-stages of a psychiatric disorder who are most likely to die by suicide, or by accidental or natural causes apparently unrelated to the primary disease process. Using chart review to obtain clinical information on these subjects has several limitations. First, the amount and detail of the available information depend upon the approach used by the clinicians recording the data and the extent to which the full chart history is actually available for review. Second, chart review is limited to subjects who have been seen by the medical profession. However, medical records can be a valuable source of information in concert with “psychological autopsies” that involve structured interviews of surviving relatives and friends and directed questioning of health care providers. Consequently, the use of psychological autopsies represents the most effective approach at present for the study of subjects who are nonelderly, noninstitutionalized, and with psychiatric disorders and comparison subjects. The value of this approach has been demonstrated by Kelly and Mann (1996) who found that comparison of research diagnoses made solely by psychological autopsy with diagnoses made antemortem by a clinician generated a kappa coefficient of 0.85 for Axis I diagnoses, a degree of agreement similar to that found in reliability studies involving direct interviews with patients. However, it may be that for certain disorders, chart review can provide a reliable research diagnosis. For example, Deep-Soboslay et al. (2005) found that for subjects with schizophrenia the agreement was high between chart review alone and interview with a surviving relative (kappa coefficient = 0.94), whereas the agreement was much lower for bipolar disorder (0.58) and major depressive disorder (0.68).

The information obtained from antemortem assessments or psychological autopsies requires formulation into consensus Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; American Psychiatric Association, 1994) diagnoses. Using an independent panel of experienced research clinicians to make these diagnoses avoids the potential biases of investigators who will be studying the postmortem tissue and provides additional parallels to the approaches that produce high diagnostic reliability in clinical studies.

**Neuropathological Assessment**

Although psychiatric disorders do not have stigmata that can be detected by standard neuropathological examination, these assessments are clearly important to exclude other diseases that may mimic the clinical features of psychiatric disorders and to characterize the presence of brain abnormalities that might affect study design or interpretation. Clearly, the types and incidence of neuropathological abnormalities are likely to differ across psychiatric subjects as a function of a variety of factors (for example, age, cause and manner of death, etc.). However, not every abnormality detected on neuropathological exam necessarily represents an exclusion condition, an important consideration given the importance of maximizing the sample sizes utilized in postmortem studies. Thus, the goal of neuropathological inquiry is to ascertain the presence of all abnormalities so that the potential impact of each can be considered in the design of each study. At the same time, it is important to minimize the impact that neuropathological assessment has on the availability of brain tissue for subsequent studies. The involvement of an interested neuropathologist in the brain collection process makes it possible to approach the assessment on an individualized basis, guided by the clinical record and gross tissue inspection to obtain standard tissue samples and additional ones as appropriate, with minimal disruption of the preferred tissue blocking approach.

**Design of postmortem studies**

The design of postmortem studies requires attention to a number of important issues that may affect outcome and interpretation, some of which are unique to these types of studies. One of the most difficult issues is whether the available postmortem tissue specimens will actually support the type of investigation under consideration. That is, the investigator must decide, independent of the interest or importance of the question to be addressed, if the characteristics of the available tissue specimens can actually provide interpretable data. If the available tissue specimens are poorly characterized or suboptimal in some respect (for example, poor ribonucleic acid [RNA] integrity), then the investigator must decide if potentially flawed or misleading data are better than no data at all. Statistical methods can be used to correct for certain differences in demographic (for example, age, sex, etc.)
or other (for example, brain pH, postmortem interval [PMI], tissue storage time, etc.) variables across subject groups. However, the matching of individual pairs (or triads) of subjects from each diagnostic group as closely as possible on these variables has several advantages. First, matching helps ensure that the mean and variance for a given independent measure will be comparable across subject groups. Second, for assays in which tissue samples from all subjects cannot be processed together, matching of subjects across diagnostic groups, and always processing tissue from matched subjects together, helps reduce the potentially confounding influence of interassay variation. Third, matching subjects makes it possible to compare the results of different statistical models, in which subject pair and/or one or more of the matching variables are entered as blocking variables or covariates (for example, see Pierri et al., 2001). Comparable results across models provide additional support for the finding of interest. However, this strategy requires the availability of a large number of subjects in the comparison groups of interest to adequately match subjects on the relevant variables.

The development of stereological methods has clearly demonstrated the importance of using systematic uniform random sampling to obtain valid estimates of the density or absolute number of objects in a region (Dorph-Petersen et al., 2004; Dorph-Petersen et al., 2007). That is, ideally the entire region of interest is sampled in a fashion such that every object of interest has an equal probability of being counted or measured. By extrapolation, estimates of the tissue concentration of a given molecule would also benefit from a similar approach to selecting the bits of tissue to be sampled. However, limitations in tissue specimens can present problems in this area so that the best possible sampling (for example, systematic and random, even if the lack of availability of the entire structure of interest prevents uniform sampling) procedure should be used, with explicit acknowledgment of the resulting limitations in interpretation. Because using a larger sample of subjects may help mitigate the problems associated with less precise measurements, the clear solution for advancing the field is to increase the availability of resources so that an appropriate number of brains is adequately sampled in all studies.

MEASURES OF INTEREST

Numerous techniques are currently used to study disease-related abnormalities in the levels of gene products in postmortem human brain samples. The first step in protein synthesis is the expression of a unique gene; this is achieved by the production of a primary RNA transcript by gene transcription that is complementary to the entire gene sequence. This primary transcript is intermediate and contains sequences encoding introns and exons. Additional processing removes the intronic sequences and the exon-complementary regions are spliced together to generate the final messenger RNA (mRNA). Following transcription, mature mRNA is translated into a protein molecule on ribosomes at the endoplasmic reticulum. Proteins then may undergo extensive post-translational modifications (glycosylation, phosphorylation, etc.), proteolysis, or assembly into protein complexes. Each of these processes has an intermediate product that can be directly measured in postmortem brain tissue; however, it is important to consider the factors that can affect those measurements in ways not related to the disease process of interest. Each technique has its strengths and limitations in the analysis of gene products that must be kept in mind in designing studies and interpreting results.

Messenger Ribonucleic Acid (mRNA)

Isolation of intact mRNA is essential for many techniques used in gene expression analysis. Northern analysis, in situ hybridization, and microarray analysis (especially when priming with oligo [dT] sequences) require RNA of extremely high integrity. Reverse transcriptase-polymerase chain reaction (RT-PCR), however, involves analysis of smaller regions of RNA (generally from fewer than 100 to 1000 nucleotides) and may be more tolerant of partially degraded RNA. A recent study, in which critical factors in gene expression in postmortem human studies were analyzed, showed that the strongest predictor of gene expression was total RNA quality (Lipska et al., 2006). Other significant factors included pH, PMI, age, and the duration of the agonal state, but their importance depended on the transcript measured, brain region analyzed, and diagnosis. Thus, it is essential to check RNA integrity before gene expression analysis in all postmortem studies (see RNA integrity estimates below) and include the other potential confounding factors as regressors in the statistical analysis of the data. Although used as a matching factor in postmortem studies of transcript expression, previous reports have shown that PMI is not predictive of RNA stability (Trotter et al., 2002; Stan et al., 2006).

Protein

Evaluating the protein product(s) of a given gene provides an important and complementary approach to assessments of the expression level of the cognate mRNA. The level of transcription of a gene gives only a rough estimate of its level of expression into a protein. An mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. In addition, many transcripts give
rise to more than one protein through alternative splicing or post-translational modifications. Immunocytochemistry, western blot analysis, radioimmunoassay, and immunoprecipitation are some of the methods used in proteomics to study the translation of mRNAs into the final gene product. These techniques use specific antibodies to assess qualitatively the cellular and regional expression patterns of proteins (for example, immunocytochemistry) or to determine quantitatively the amount of protein in a given sample (for example, western blots). Other protocols, such as autoradiography, use specific ligands which by binding to proteins (for example, receptors) can provide quantitative data on the expression and functional integrity of the targeted molecules.

A special consideration for immunocytochemistry in postmortem brain studies is the method of tissue fixation, which prevents the diffusion and degradation of the protein to be detected. Immunoreactivity can be affected by the type of fixative and by the duration of fixation. Antigen-retrieval techniques, such as exposure of tissue specimens to elevated temperature or microwave irradiation, have produced remarkable recovery of immunoreactivity (Rangell and Keller, 2000). However, limited data exist about whether this recovery is consistent across samples with different fixation exposure times. It is also important to note that fixation and other processing and storage conditions of tissue can produce substantial tissue shrinkage (Dorph-Petersen et al., 2005) that may be difficult to assess or account for, depending upon the type of study to be conducted, and that can substantially bias measures of regional brain volumes or density of neural structures. Interestingly, no studies to date have examined whether the amount of tissue shrinkage differs as a function of diagnosis.

Many proteins undergo post-translational modifications that profoundly affect their activities; for example, some proteins are not active until they become phosphorylated. Phosphoproteomics and glycoproteomics, which assess these post-translational modifications, are currently being evaluated in postmortem human brain research. However, the phosphorylation state of proteins can change very rapidly after death (Li et al., 2003), raising concerns about the potential yield of such studies. Most proteins function in collaboration with other proteins, and one goal of proteomics is to identify which proteins interact. Methods to probe protein-protein interactions include protein microarrays, immunoaffinity chromatography followed by mass spectrometry, and combinations of experimental methods such as phage display and computational methods. For example, two recent papers assessed the functional integrity of proteins and the activation of their signaling cascades in postmortem tissue (Hahn et al., 2006; Dwivedi et al., 2007). In these kinds of studies, the protein extracts from the postmortem tissues need to be prepared under conditions in which protein-protein interactions are maintain, which requires, among other things, the availability of tissue samples in which protein degradation is limited. Furthermore, the degree to which the results of these protocols correspond to in vivo measurements is unclear.

**POTENTIAL CONFOUNDS**

Premortem, perimortem, and postmortem factors can affect the quality of the results in mRNA and protein detection studies in human brain research. Thus, it is essential to determine as fully as possible the presence of such factors and to systematically assess their potential impact on the biological measures of interest.

**Premortem Factors**

Comorbid conditions, such as alcohol, nicotine, or other substance abuse, are common in many psychiatric disorders. Prevalence of current abuse/dependence in psychiatric inpatients ranges from 12%–60% (Brady et al., 1991; Havassy and Arns, 1998; Cantwell et al., 1999), and from 48%–64% for lifetime abuse/dependence (Brady et al., 1991; Dixon et al., 1991). Among outpatients, rates of lifetime and current abuse/dependence vary from 6%–60% (Gogek, 1991; el Guebaly and Hodgins, 1992; Fowler et al., 1998). In particular, the lifetime prevalence rate of substance abuse among people with schizophrenia is close to 50% (Dixon et al., 1991; Mueser et al., 1995), with prevalence rates for current substance abuse as high as 65% in some samples (Mueser,Bellack, et al., 1992; Mueser, Yarnold, et al., 1992). Similarly, individuals with schizophrenia and bipolar disorder have extremely high rates of nicotine use, 2 to 3 times higher seen in the general population and considerably higher than among subjects with other psychiatric illnesses. In the United States about one fourth of the population are smokers (Hymowitz et al., 1997), whereas more than 70% of patients with schizophrenia are nicotine dependent (Ziedonis and George, 1997; Van Dongen, 1999). Among other drugs, cannabis and cocaine are the most commonly used in the dual-diagnosis population (Soyka et al., 1993; Dixon et al., 1991; Hambrecht and Hafner, 2000; Duke et al., 2001).

Each of these comorbid conditions can affect measures of interest in postmortem studies. For example, drugs of abuse such as cocaine and amphetamine produce an experience-dependent structural plasticity that alters dendritic length and complexity in certain brain areas (Robinson et al., 2001; Robinson et al., 2002; Crombag et al., 2005). Alcohol abuse and smoking have documented effects on the expression of specific gene
products (Liu et al., 2004; Flatscher-Bader et al., 2005; Flatscher-Bader and Wilce, 2006; Flatscher-Bader et al., 2006). Chronic alcoholism affects a number of signaling cascades and transcription factors, which in turn result in distinct gene expression patterns, such as pronounced differences in expression of myelin-related genes and genes involved in protein trafficking in the cerebral cortex (Liu et al., 2004). Significant changes in the expression of known alcohol-responsive genes, and genes involved in calcium, cyclic adenosine monophosphate (cAMP), and thyroid signaling pathways have also been identified (Mayfield et al., 2002). Nicotine administration also modulates the expression level of a variety of genes, including those involved in transcriptional activation and their catecholamine and neuropeptide synthesis. In animal models, chronic exposure to nicotine induces long-term increases in the mRNA expression levels of genes involved in the regulation of food intake and energy expenditure, such as neuropeptide Y (NPY), orexins, and their receptors (Kane et al., 2000; Li et al., 2000).

The exclusion of all subjects with any history of a substance abuse disorder, though seemingly ideal from the perspective of eliminating the impact of confounding factors, is problematic from other perspectives. For example, the high incidence of these confounds in the subject populations of interest, and the limited availability of postmortem tissue, means that the resulting studies would likely have very small sample sizes. In addition, the resulting sample would be highly biased to only a certain subpopulation of individuals with the diagnosis of interest. Thus, a better alternative is the comparison within and across diagnostic groups of subjects with and without comorbid substance use histories. Within the diagnostic group of interest, if the subgroups with and without substance use do not differ on the measure of interest, and both show similar divergence from a normal comparison group, then one can provisionally conclude that the comorbid factor does not influence the measure of interest, and thus that the observed alteration reflects the underlying disease process.

Clearly, assessment of the potential impact of psychotropic agents in postmortem studies requires knowledge of the presence of such agents. At present, this assessment is usually limited to assays of blood or other bodily fluids. The extension of such evaluations to brain tissue and hair would, respectively, provide data that may be more biologically relevant and that would reflect exposure over the several months preceding death.

Similarly, the interpretation of findings from a postmortem study requires determining whether the measure of interest is influenced by the pharmacological treatment of the illness under investigation. This determination depends upon knowledge of which medications the subject received, for how long, and at what dosage. However, such detailed information is difficult to obtain, frequently incomplete, confounded by adherence issues, and usually not available to the same degree across subjects (with the possible exception of individuals who have been chronically hospitalized). Even when such information is available, it is difficult to know how to make comparisons across subjects: for example, is the relevant measure dose at time of death, total life time dose, and so on? Finally, a substantial percentage of subjects have received more than one psychotropic medication, and the extent to which drug interactions confound the picture is difficult to assess.

Because adequate samples of postmortem brain specimens from subjects who were never medicated with a given psychiatric disorder are not available, several less direct approaches must be used to address the influence of psychotropic medications. These approaches include (1) the comparison of data from subjects who were on or off the medications of interest at the time of death; (2) the examination of subjects with other disorders who were treated with the same medications; and (3) the use of animal models that mimic the clinical treatment of the disorder under investigation.

Certainly, the first two approaches have obvious, and difficult to control, potential confounds. Long-term exposure to psychotropic medications, as is typical in the treatment of most psychiatric disorders, may have effects on brain morphology, neurochemistry, or gene expression that persist for a substantial period of time after the drug is discontinued. In addition, even though the same agents are utilized, the pharmacological treatment of other disorders may be different than that of the disease of interest. For example, compared with schizophrenia, the treatment of other psychotic disorders with antipsychotic medications tends to be more intermittent or for a shorter duration of time.

In the case of animal models of drug effects, studies in rodents frequently involve dosage and/or length of treatment parameters that do not reflect the human treatment condition. These limitations can be overcome through studies in nonhuman primates that involve extended periods of treatment with medication-dosing regimens that produce serum drug levels demonstrated to be therapeutic in humans. For example, chronic exposure to antipsychotic medications, in particular haloperidol and olanzapine, in macaque monkeys was associated with an 8%–11% reduction in mean fresh brain weights, with the differences between treatment conditions most robust in the frontal and parietal lobes (Dorph-Petersen et al., 2005). However, the potential problem of species differences, and the possibility that the medications of interest may have different effects on the brain of an individual with a psychiatric illness than on the normal brain, must be kept in mind. Despite the limitations of each of these three approaches individually, convergent findings across them should lead to reasonable conclusions about the extent to which psychotropic medications account for any observed brain
differences between subjects who are psychiatrically ill and normal comparisons.

Perimortem Factors

The manner and cause of death also have potential influences on the measures of interest. For example, in cases of prolonged agonal state and terminal stress, conditions such as hypoxia or ischemia activate anaerobic metabolic pathways, predominantly the lactic acid cycle, resulting in an increased production of acid equivalents, responsible for inter- and intracellular acidosis (Rehncrona et al., 1985; Yates et al., 1990). Evidence from analyses of postmortem human brain has shown that brain tissue pH is decreased by prolonged agonal states accompanied by hypoxia (Hardy et al., 1985; Kingsbury et al., 1995). Furthermore, a recent study suggests that increased levels of lactate, possibly due to antipsychotic medications, are associated with decreased pH in postmortem human brains of patients with schizophrenia (Halim et al., 2007). On the other hand, brain tissue pH appears to remain constant across postmortem delays (Kingsbury et al., 1995) and has also been reported to be unaffected by freezer storage time (Stan et al., 2006). Brain tissue pH also seems to be similar across different regions within a given brain (Harrison et al., 1995; Mexal et al., 2005; Mexal et al., 2006). Although protein immunoreactivity and ligand binding to receptors show no change related to brain pH (Harrison et al., 1995), brain pH does predict enzymatic activity (Perry et al., 1986; Yates et al., 1990), and mRNA integrity (Harrison et al., 1995; Kingsbury et al., 1995; Johnston et al., 1997; Bahn et al., 2001; Tomita et al., 2004).

Interestingly, a recent microarray study revealed a functional specificity of changed genes related to pH. With lower pH values, the expression of transcripts encoding stress-response proteins and transcription factors was altered, suggesting that the change in their tissue levels is not simply due to an overall RNA degradation in response to low pH but reflected a biological response in living cells in response to stress (Li et al., 2004; Vawter et al., 2006).

Reliable RNA expression and protein data can be obtained from postmortem brains with relatively long PMIs (see below) if the agonal factors and acidosis are not severe. Although pH values are correlated with RNA integrity, a higher pH does not guarantee intact RNA. For example, an analysis of a large brain collection revealed that several diagnostic groups had significantly lower pH values than other groups, but they did not have significantly lower RNA integrity (Webster, 2006). Consequently, RNA integrity must be assessed for every case before it is included in a postmortem study.

Measurement of mRNA levels in postmortem tissue is based on the assumption that these levels reflect the amount of transcripts in vivo. The kinetics of degradation of newly synthesized cytoplasmic poly(A)-bearing RNA have been examined in resting human lymphocytes (Berger and Cooper, 1975). Two classes were identified, a labile component with a short half-life and a very stable component that remains undiminished across long time periods. These findings raise the possibility that partial degradation could cause a variable bias in the quantification of different transcripts (Auer et al., 2003). Furthermore, in the case of postmortem samples, the integrity of RNA molecules results from a combination of circumstances that can include in vivo, in situ and in vitro events. Indeed, active exo- and endonuclease activity may occur in live tissue during early postmortem periods, and autocatalytic degradation generates random fragmentation at later times in dead tissue or during extraction/storage of RNA material.

18S/28S ratio

Because mRNA comprises only 1%-3% of total RNA, it is difficult to detect. Ribosomal RNA (rRNA), on the other hand, makes up more than 80% of the total RNA sample, with the majority of that comprising the 28S and 18S rRNA species. Messenger ribonucleic acid quality traditionally has been assessed by electrophoresis of total RNA followed by staining with ethidium bromide. This method relies on the assumption that rRNA quality and quantity reflect that of the underlying mRNA population. According to this, intact total RNA run on a denaturing gel will have sharp, clear 28S and 18S rRNA bands (Fig. 15.1, Panel A). The 28S rRNA band should be approximately twice as intense as the 18S rRNA band.

With increasing degradation, heights of 18S and 28S peaks gradually decrease and additional “degradation peak signals” appear in a molecular weight range between small RNAs and the 18S peak (Fig. 15.1, Panel B). The ratio of the average degradation peak signal to the 18S peak signal multiplied by 100 is referred to as the degradation factor. This analysis has been tested on 19 tissues of seven organisms, and it is a reproducible parameter for degradation of mammalian RNA (Auer et al., 2003).

A 2:1 ratio (28S:18S) is a good indication that the RNA is completely intact. Because mammalian 28S and 18S rRNAs are approximately 5 kb and 2 kb in size, the theoretical 28S:18S ratio is approximately 2.7:1; but a 2:1 ratio has long been considered the benchmark for intact RNA. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit the 2:1 ratio of high-quality RNA. Completely degraded RNA will appear as a very low molecular weight smear (Fig. 15.1, Panel A).

Isolating RNA from human tissue presents challenges that are not always present in experimental animal work. Confounding factors almost guarantee that human total RNA will rarely have a 28S:18S rRNA ratio of 2:1.
Although crisp 28S and 18S rRNA bands are indicative of intact RNA, it is less clear how these long-lived and abundant molecules actually reflect the quality of the underlying mRNA population, which turns over much more rapidly.

A drawback to using denaturing agarose gels to assess integrity RNA is the amount of RNA required for visualization. Generally, at least 200 ng to 1 µg of RNA must be loaded onto a denaturing agarose gel to be visualized with ethidium bromide. Some small RNA preparations, such as those from laser capture microdissected samples, produce very low yields. In these cases, it may be impossible to spare 200 ng of RNA to assess integrity before proceeding with the expression profiling application. Alternative nucleic acid stains, such as SYBR Gold and SYBRi Green II RNA gel stain from Molecular Probes (Eugene, OR), offer a significant increase in sensitivity compared to the traditional ethidium bromide stain in agarose gels.

**RNA integrity number (RIN)**

An alternative to traditional gel-based analysis that integrates the quantification of RNA samples with quality assessment in a quick and simple assay uses a combination of microfluidics, capillary electrophoresis, and fluorescent dyes that bind to nucleic acid to simultaneously evaluate RNA concentration and integrity. Automated determination of RIN has provided a direct measure of RNA quality requiring as little as 10 ng of RNA per analysis. In addition to assessing RNA integrity, this automated system also provides a good estimate of RNA concentration and purity (that is, rRNA contamination in mRNA preparations) in a sample. In this system, an integrity number is automatically assigned to a eukaryote total RNA sample. The 28S:18S rRNA ratio is calculated by integrating the areas of 18S and 28S rRNA peaks and then dividing the area of the 18S rRNA peak into the area of the 28S rRNA peak. Using this tool, sample integrity is no longer determined by the ratio of the ribosomal bands, but by the entire electrophoretic trace of the RNA sample. This includes the presence or absence of degradation products. In this way, interpretation of an electrophorogram is facilitated, comparison of samples is enabled and replication of experiments is ensured. The assigned RIN is independent of sample concentration, instrument, and analyst and therefore

---

**FIGURE 15.1** Ribonucleic acid (RNA) integrity profile. (A) Virtual gel from human brain RNA samples run on an Agilent 2100 Bioanalyzer. 28S and 18S ribosomal RNAs are clearly visible in high quality samples (lanes 1, 2, 4, 5, 7, and 8; 28S/18S ratios in 1.30 to 1.54 range; RNA integrity number [RIN] in 7.3 to 8.5 range). Lanes 3 and 6 show degradation with shifts towards lower molecular weight and laddering appearance (Lane 3; 28S/18S n/a; RIN = 3; Lane 6, 28S/18S = 0.51; RIN = 5.7). Lane L, molecular weight markers. Low molecular weight RNA species are present in the lower part of the gel lanes. (B) Bioanalyzer chromatograms of RNA samples with decreasing 28S/18S ratio (values indicated in graphs). Note the progressive shift to the left in peak heights from 18S and 28S to lower molecular weight with decreasing RNA quality.
represents a legitimate standard for RNA integrity. It correlates with tissue pH and incorporates the extent of RNA degradation with the presence of 28S/18S peaks (Ross et al., 1992; Colangelo et al., 2002; Jones et al., 2006) (Fig. 15.2, Panel B). However, there is a limitation in the use of RIN as an indicator of mRNA quality. Under certain circumstances, some specific transcripts show special degradation rates (Buesa et al., 2004; Barrachina et al., 2006); this suggests the necessity of verifying individual mRNA quality within an experiment.

3′/5′ ratio

3′/5′ ratios are measurements of RNA quality that relate to specific technical aspects of oligonucleotide microarrays.

Deoxyribonucleic acid microarray technology relies on the monitoring of relative changes in RNA abundance between samples. Microarrays investigate changes in transcriptomes (the set of all expressed mRNA expressed in a tissue sample), and results are therefore particularly dependent on RNA quality. Here we briefly describe how 3′/5′ ratios are generated, how they are influenced by RNA quality, and how 28S/18S ratios and RIN numbers correlate with 3′/5′ ratios and can predict the quality of array results.

The array technology is essentially an extension of southern hybridization, in which the labeled probe is free in solution and the target mRNA sequence is fixed on a solid phase. To generate probes corresponding to expressed mRNAs, total RNA is reverse-transcribed and converted into double-stranded complementary...
deoxyribonucleic acid (cDNA), using primers that are complementary to the poly(A) tails located at the 3′ end of mRNAs (Fig. 15.2, Panel A). The length of the labeled probes will depend on the integrity of the RNA template and on the reverse transcription and amplification steps (Fig. 15.2, Panel A). Thus, this labeling protocol is sensitive to 5′-end degradation and internal fragmentation. For instance, fragmented mRNA will limit the extent of reverse-transcription and yield short labeled probes, while intact mRNA will allow for longer probes to be generated. Oligonucleotide microarrays from Affymetrix, Inc (Santa Clara, CA) have incorporated in their array designs different sets of targets for several control genes. These different targets are located at different distances from the 3′-end of the respective control mRNAs. Differences in intensity of hybridization at proximal (3′) versus distal (5′) probes can therefore be used to estimate the proportion of intact to truncated or shortened RNA templates, as the probability of an internal break being present in the template increases with the distance of the target from the 3′-end poly(A) tail. Targets have been designed for the actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes so that values for 3′/5′ ratios that are close to one correspond to optimal RNA integrity for array experiments, while ratios higher than 3 are indicative of levels of RNA degradation that will compromise the reliability of the array results.

**Postmortem Factors**

Factors that occur after death can also affect the measures of interest in postmortem human studies. For example, the PMI, the elapsed time between death and the freezing or immersion of brain tissue in fixative, is a frequently employed measure of the quality of postmortem brain specimens. The effect of PMI on a given dependent variable may be complex, and it is not always the case that a short PMI ensures a valid assessment of the measure of interest. The nonlinear nature of PMI effects has been demonstrated in several studies. For example, a study of the impact of PMI on the somal size of a specific subtype of interneurons in adjacent blocks of monkey neocortex revealed that PMI predicted somal size between 30 minutes to 12 hours, but not between 12 and 48 hours (Hayes et al., 1991). The tissue concentrations of certain members of a family of proteins may also change in different ways as a function of PMI. Figure 15.3 compares the tissue concentrations of three prosomatostatin-derived peptides in biopsy samples of monkey prefrontal cortex frozen at different time intervals after removal, creating a range of PMIs (Hayes et al., 1991). The concentration of one peptide, somatostatin-28, declined to 10%–20% of baseline levels at the 10-minute PMI, whereas the concentrations of two other peptides, somatostatin-14 and somatostatin-28 (1-12), actually increased during the same time interval, presumably as a consequence of the cleaving of somatostatin-28. However, by 12-hour PMI, each of these peptides showed a different relative tissue concentration compared with its baseline level. That is, somatostatin-28 levels remained stable at about 20% of baseline, somatostatin-14 levels were approximately 60% of baseline, and somatostatin-28 (1-12) levels had returned to baseline levels. Similar complex, PMI-related effects have also been observed for different isoforms of the same protein (Lewis et al., 1993, 1994).

Postmortem interval may also have a differential effect on immunoreactivity for the same protein in different brain structures, and even across layers within the same region of the cerebral cortex. For example, an antibody directed against nonphosphorylated epitopes of neurofilament proteins (NFP) identifies a subpopulation of neurons in the human entorhinal cortex with characteristic regional and laminar patterns of distribution (Beall and Lewis, 1992). In the intermediate subdivision of the human entorhinal cortex, intensely immunoreactive NFP-positive neurons are present in layers 2 and 5 (Fig. 15.4, panel A). Interestingly, in a pilot study of schizophrenia, NFP-immunoreactive neurons were absent in layer 2 but were clearly detectable in layer 5 (Fig. 15.4, panel B), suggesting the possibility of laminar- and neuron-specific disturbance in this illness (Lewis and Akil, 1997). However, in adjacent thin tissue blocks of monkey entorhinal cortex fixed at varying PMIs, NFP-immunoreactive neurons were clearly present in layers 2 and 5 following a 30-minute PMI (Fig. 15.4, panel C), but in layer 2 the overall

![Figure 15.3](image-url)
intensity of immunoreactivity was substantially reduced at 12-hour PMI and was undetectable following a 24-hour PMI (Fig. 15.4, panel D). In contrast, layer 5 neurons remained clearly immunoreactive up to a 48-hour PMI. These findings demonstrate that PMI effects on the same protein can differ across cortical layers within the same brain region, and they illustrate how such complex effects could be misinterpreted as the result of a disease-specific process.

Freezer storage time for fresh-frozen tissue has been reported to be negatively correlated with tissue levels of some, but not all, mRNA transcripts (Burke et al., 1991; Harrison et al., 1995). However, immunoreactivity does not appear to be altered in tissue fixed for a standard period of time and then stored in cryoprotectant at -30°C (Erickson et al., 1998).

CONCLUSIONS

Postmortem human brain research is a fundamental component in the study of the neurobiology of mental disorders and is essential in characterizing potential drug targets for treatment of these conditions. Two of the most critical variables in selecting the subjects for inclusion in a postmortem study are diagnostic verification and exclusion of brains with coexisting pathology or confounding factors that will severely limit the investigation of the primary disease pathology. The next step is to evaluate the integrity of the biological material. New RNA integrity measures have become available, like RIN and 3‘/5‘ ratio, and offer a more reliable and accurate evaluation of postmortem tissue quality. Together, the multiple confounds in postmortem research require

FIGURE 15.4 Brightfield photomicrographs comparing nonphosphorylated neurofilament protein (NFP) immunoreactivity in the intermediate subdivision (EI) of human entorhinal cortex from a normal control subject (A) and a subject with schizophrenia (B). Note the absence of NFP immunoreactivity in layer II clusters of neurons in the subject with schizophrenia, despite the presence of these neurons on an adjacent Nissl-stained section (not shown) and the presence of intensely immunoreactive neurons and dendrites in the deep cortical layers, similar to that of the normal control subject. However, the decreased NFP immunoreactivity in layer II of panel A appears to represent a postmortem effect rather than a schizophrenia-related change as indicated by the result of experiments summarized in panels C and D. These panels show NFP immunoreactivity in sections from adjacent tissue blocks, containing the same subdivision of monkey entorhinal cortex, that were processed following postmortem intervals of 0.5 (C) and 24 (D) hours. Note that the longer postmortem interval (PMI) (D) results in a loss of NFP immunoreactivity that is restricted to layer II. Scale bar = 150 µm and applies to all panels. Figure reprinted from Lewis and Akil (1997).
the use of convergent approaches to explicate and minimize the potential influence of these confounds. The use of animal models in parallel studies provides one means to assess potential confounds in a systematic fashion.

In summary, reliable postmortem studies are based on an appropriate understanding of potential confounds prior to the experimental procedure and the inclusion of well-designed controls to assess the potential impact of confounds on the measures of interest. Appropriate tissue preparation allows the maintenance of the structural and molecular integrity of postmortem tissue permitting detailed morphological and molecular investigations. Continued advances in the estimation of tissue quality and the impact of confounds will help increase the potential clinical value of the observations made through the direct investigation of the postmortem human brain.

ACKNOWLEDGMENTS
Cited work conducted by the authors was supported by National Institutes of Health Grants MH045156, MH043784 and MH067721, and by a National Alliance for Research on Schizophrenia and Depression (NARSAD) Young Investigator Award.

REFERENCES


