

# Gene Expression Profiling with DNA Microarrays: Advancing our Understanding of Psychiatric Disorders

**Julie Pongrac,<sup>1</sup> Frank A. Middleton,<sup>2,6</sup> David A. Lewis,<sup>1,3</sup> Pat Levitt,<sup>1,2,5</sup> and Károly Mirnics<sup>1,2,4,7</sup>**

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DNA microarray transcriptome profiling of the postmortem brain opens novel horizons in understanding molecular changes associated with complex psychiatric disorders. With careful analysis and interpretation of microarray data we are uncovering previously unknown, expression patterns that maybe subject-specific and pivotal in understanding the disease process. In our recent studies, analyses of the prefrontal cortex of subjects with schizophrenia and matched controls uncovered complex changes in the expression of genes related to presynaptic secretory release, GABAergic and glutamatergic transmission, metabolic pathways, myelination, as well as cAMP and phosphoinositol second messenger systems. Our goal will be to integrate this expression data within the context of the relevant anatomical, biochemical, molecular, imaging and clinical findings.

**KEY WORDS:** Gene; expression; profiling; microarray; human; psychiatric.

## The Challenge: Understanding the Neural Basis of Psychiatric Disorders

Severe mental illnesses, including schizophrenia, bipolar disease, depression, generalized anxiety, and obsessive compulsive disorder, are common across different cultures throughout the world. Together, they ac-

count for an extremely high proportion of the global burden of disease, especially in developed countries (1,2). Despite the prevalence of these disorders and our sustained effort to understand their neuropathological and molecular underpinnings, much remains to be done in defining the genetic liabilities and key environmental stimuli that lead to the development of these diseases. The introduction of molecular technologies for the identification of receptor subtypes has significantly contributed to the evolution of pharmacotherapies for psychiatric disorders and consequently to our understanding of their underlying pathophysiological mechanisms (3,4). However, pharmacotherapeutic treatments remain less than ideal. Thus, improving the efficacy and specificity of the pharmacotherapy of psychiatric disorders remains a long-term priority. Indeed, the positive symptoms like hallucinations in schizophrenia can be managed even with classical antipsychotic agents such as haloperidol. However, these same agents are less effective at managing the negative, affective and cognitive symptoms associated with this illness (5–9), which are considered to be stronger predictors of the overall

<sup>1</sup> Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

<sup>2</sup> Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

<sup>3</sup> Department of Neuroscience, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

<sup>4</sup> Department of PittArray Core, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

<sup>5</sup> John F. Kennedy Center for Human Development, Vanderbilt University, Nashville, TN 37203.

<sup>6</sup> Department of Neuroscience and Physiology, Upstate Medical University, Syracuse, NY 13210.

<sup>7</sup> Address reprint request to: Károly Mirnics, M.D., Department of Psychiatry, University of Pittsburgh School of Medicine, W1655 BST, Pittsburgh, PA 15261. Tel: (412)648-9788; Fax: (412)648-1441; E-mail: karoly+@pitt.edu

prognosis for the patient (10). Second generation atypical antipsychotic medication such as clozapine, olanzapine, risperidone, and ziprasidone may be more effective against the negative and cognitive effects of schizophrenia, and produce reduced extrapyramidal effects, than the typical neuroleptic drugs (11–17). Still, atypical neuroleptic drugs produce side effects such as weight gain (17–22) elevated serum lipids (20) and an increased risk of diabetes (23). In addition, little is known about how these effects influence long-term mortality, morbidity and compliance to therapy (24). Finally, despite the improvements associated with atypical neuroleptic drugs, at least 20–30% of patients remain refractory to treatment (25–27). The problems associated with the currently available treatments for schizophrenia are not unlike those for the other psychiatric disorders: treatment of major depressive disorder with currently available antidepressants is complicated by non-specific side effects and variability in individual responsiveness, tolerability, and compliance (28).

Today, as the search for novel drug targets moves beyond the receptor level to the identification of aberrant intracellular signaling pathways and alterations in gene expression, there is an increasing need for analyses of the *transcriptome*—the collection of all expressed mRNAs by a cell, tissue, or structure. This transcriptome is the product of genomic DNA and is one of the key determinants of cellular phenotype, function and response to environmental variables (29–32). Several modern and comprehensive technologies are now available for the study of transcriptomal patterns, and we are witnessing their power in identifying neural substrates that play a role in the pathology and potential treatments for mental disorders.

### An Array of Arrays: Platforms for Microarray Gene Expression Profiling

Microarrays, amongst other functional genomics methods (subtraction by hybridization, SBH; serial analysis of gene expression, SAGE; total gene expression analysis, TOGA; massively parallel signature sequencing, (MPSS) (34–37), are at the forefront of this technological revolution. These technologies take advantage of information that has been deposited into sequence databases as gene sequences and expressed-sequence tags (ESTs) (38–40). DNA microarrays are high-density arrangements (often >10,000 genes) of complementary DNA sequences (cDNA) or synthetic oligonucleotides immobilized in specific locations on a

solid surface or membrane (38–40). Each microarray “probe” is capable of recognizing complementary sequences through base pairing—the process of hybridization (41). These DNA arrays are successfully used for multiple purposes, including genotyping, comparative genomic hybridization, sequencing and expression analysis (39,40,42,43). In analysis of gene expression, the whole labeled sample, or “target” (DNA or RNA) is hybridized to the immobilized DNA sequence probes on the array surface. After a series of high-stringency washes only the highly complementary probe-target complexes remain tightly bound, and the amount of the retained label is analyzed over each DNA probe spot (41,44). The amount of the retained label is a measurement of the probe-target interaction—and the abundance of the mRNA species in the sample. Different DNA array platforms exist and each have specific advantages and weaknesses that are essential to consider in order to achieve a sound experimental design (Refs. 45–47 for review).

*cDNA Microarrays.* They are made by deposition of long cDNA probes (usually >100 bp to ~2 kb), either by mechanical microspotting, ejection, or ink-jetting techniques onto a solid support (30,33). The cDNA probes are usually PCR-amplified DNA clones or ESTs derived from the 3'-end of RNA targets and arrayed onto a glass or membrane (30,33). cDNA array experiments are most often performed as dual fluorescence experiments (39,48,49). If fluorescent makers are used, multiple targets (test and reference samples) can be labeled with separate fluorochromes (often Cy3 or Cy5), combined, and hybridized together onto the same microarray that contains thousands of probes. Using two independent scans under the incorporated fluorochrome's emission wavelengths, a high-resolution fluorescent intensity measurement is obtained from each DNA spot on the microarray. This signal is quantified, standardized and compared between the two fluorescent images, and the intensity differences are interpreted as transcript abundance differences between the two compared samples. In contrast, radioactively labeled targets may be used to generate a stronger signal derived from the hybridization of cDNA with targets (40–53), but test and reference samples are analyzed in parallel hybridizations on different microarrays. Consequently, this approach may result in increased experimental variability.

The earliest microarray gene expression profiling studies were conducted on custom designed cDNA microarrays developed by individual research groups (38–40,48,49,54–56). Based on the relatively low cost,

great flexibility and customizable probe sets, this approach of transcript analysis remains popular to date (29). Indeed, many institutions have developed core facilities with the necessary robotic printers and laser-based confocal scanners to accommodate the efforts of independent research groups (31). However, the new generation of reasonably priced, commercially available, high-density DNA microarrays are becoming a convenient choice, particularly for investigators who have limited molecular biology experience.

**Synthetic Oligonucleotide Probe Arrays.** These arrays are high-density, two-dimensional arrays of synthetic oligonucleotides that are currently manufactured under a proprietary technology of Affymetrix (40,57). These GeneChip expression arrays are glass-supported arrays derived by spatially arranging light-directed combinatorial chemical synthesis of thousands of different oligonucleotides (32). This photochemical, solid-phase technology facilitates the high-spatial resolution and precision of probe placement that consequently provides the very high-density throughput of gene expression analyses. The recently introduced human U133 Genechip contains features (probe spots) as small as  $18 \times 18 \mu\text{m}$  on a single  $1.28 \times 1.28 \text{ cm}$  array and represents up to 80% of the human genome across two arrays (<http://www.affymetrix.com/products/arrays/specific/hgu133.affx>). The expression of each gene is interrogated with multiple probes, and the “transcript presence” is detected by comparing the control (“mismatches”) and experimental (“perfect match”) features using an advanced mathematical modeling process. Similarly, after a routine standardization, expression differences across different arrays (different samples) are detected using a statistical algorithm.

Synthetic oligonucleotide microarray technology should become increasingly affordable, and therefore available to more investigators, due to exciting advancements in combinatorial chemical processing. One such advance has been the development of digital light processors that will make possible maskless oligonucleotide synthesis and the production of high-density and customizable oligonucleotide arrays at the bench top in the near future (57–59). The recent collaboration of Agilent laboratories, the Defense Advanced Research Projects Agency (DARPA) and the University of Colorado to develop a chemical process that requires two rather than four steps in the synthesis of nucleic acids is another such advancement ([http://www.labs.agilent.com/news/2001features/fea\\_darpa.html](http://www.labs.agilent.com/news/2001features/fea_darpa.html)). This technology promises to improve the safety and efficiency of the microarray production process rendering

it more amenable to the production of DNA microarrays that accommodate genome-wide transcriptome interrogation of biological samples.

**Spotted Oligonucleotide Arrays.** They represent a recent evolution of the classic cDNA arrays. Oligonucleotide probes of 50–80 nucleotides are synthesized on a large scale and in a secondary process spotted onto surface-activated glass slides. These oligonucleotides are often chemically modified to facilitate a covalent attachment to the arraying surface. Recent introduction of “ready to print” commercial amino-modified oligonucleotide sets (Operon, <http://www.operon.com/>; MWG-Biotech, <http://www.mwg-biotech.com/>) offer a convenient option for microarray gene expression profiling. Using the spotting techniques of cDNA microarrays, they can be used to fabricate customized microarrays on epoxy-coated slides (e.g., Pan epoxy slides, MWG-Biotech) that covalently bind the amino-modified oligonucleotides. Spotted oligonucleotide arrays work well in dual fluorescent hybridizations, and often can be successfully used for distinguishing between splice variants of the genes. Oligonucleotide arrays can also be printed on different substrates, which may offer increased sensitivity and reproducibility (e.g., CodeLink platform).

**cDNA Macroarrays.** These are the forgotten, non-fashionable, but very sound and low-cost alternatives for transcriptome profiling (45,50–52,60). The macroarrays are printed on nylon or nitrocellulose membranes and may contain as many probes as high-density microarrays. Processed with  $^{32}\text{P}$  or  $^{33}\text{P}$  sample labeling in a standard reverse transcription, this procedure requires small amounts of material and produces very reliable and reproducible data. Array images are obtained using phosphorous screens, and they are analyzed in a similar fashion as cDNA array images. Recent evolutions of this technology include nitrocellulose-coated glass slides that can be processed both using a radioactive or fluorescent label (52).

### Expression Profiling the Human Brain Using Microarrays: What Matters?

Microarray profiling of the human brain has its specific requirements, both in performing the experiment and in interpreting the data (45,61). The use of postmortem tissue as starting material, sample matching paradigms, specificity of the observed changes and reproducibility across cohorts are only a few selected issues that will be briefly discussed.

**Postmortem Tissue.** This tissue is generally an excellent source of mRNA (62–64). Transcript integrity seems to primarily depend on brain pH (which reflects the degree and duration of hypoxia and acidosis prior to death), tissue handling and RNA isolation procedures (63,64). Postmortem interval up to 40 hours alone does not seem to account for observed gene expression changes. In our microarray and *in situ* hybridization experiments we have not observed significant effects of PMI and brain storage time on gene expression levels. Furthermore, on Affymetrix microarrays 3' vs. 5' mRNA integrity ratios are routinely comparable to those seen involving rodent brain with a PMI of 2 minutes (3':5' integrity ratio = 1:1.2).

**Sample Matching Paradigms.** These require different approaches for the distinct microarray platforms and should take into account the specific experimental design employed. While limited pooling of tissue may reduce individual variability in animal experiments where the experimental variables and genetic differences are controlled for, pooling tissue from subjects with complex, multigenic disorders with a high degree of “individuality” (e.g., schizophrenia, major depression) is of little value. The tissue of each human subject is unique, and its gene expression is shaped not only by genetic but also epigenetic factors, including lifestyle and treatment of the disease. Regardless of the type of analyses performed, the experimental-control groups (and pairs within the groups) when investigating brain disorders of human subjects should be matched for age, race, PMI, gender, agonal state and as many comorbid factors as possible.

In single channel hybridizations (e.g. GeneChip, CodeLink, membrane arrays) each sample is hybridized to a separate array. Transcriptomes across multiple arrays are normalized for interarray variability using spiked-in standards or overall signal intensity. After a standardization step, the results obtained may be compared group-wise or pairwise. Unfortunately, in group-wise analyses less robust data may be lost in the multiple standardization steps.

When DNA arrays are used in dual-fluorescence experiments, two options are available. The first option is a direct hybridization of a best matching pair (control vs. experimental sample) compared on the same array. This experiment, whenever possible, should be also performed with reverse labels (65) to compensate for dye incorporation bias. This approach has an advantage that it compensates for interarray variability, but the standardization and data analysis across arrays becomes difficult due to lack of a common reference. The second option is to hybridize the individual ex-

perimental and control samples against a common reference. This reference is later used to standardize all the samples participating in the experiments. Using a reference sample has an advantage that allows post hoc cross-array comparisons, but it may be less sensitive than direct sample comparisons.

**Specificity of Observed Expression Changes.** Specificity is critical for interpretation of the data. Microarray results across several diseases and animal models suggest that different diseases and conditions may share many adaptational gene expression changes. The vast majority of these changes will likely represent a non-specific response of the CNS tissue to different pathophysiological processes. As a result, establishing whether the same pattern of expression is present in a different disease is important for the interpretation of the findings.

**Reproducibility of Findings.** The reproducibility is essential across different cohorts. Data obtained on a limited number of investigated subjects may be true and valid, yet it may be a characteristic of only that cohort of subjects, and not of the disease process in general (66). This is especially common in multifactorial and multigenetic disorders, where the expression profile of the disease sorts along a wide continuum (67). We need large sample sizes to understand complex gene expression patterns, and although today we may not be able to synthesize all the data available from different sources, in the future, studies across cohorts will be combined and it will help us define the transcriptome phenotypes associated with the disease (61,68–71).

### Changes in Gene Expression: Genetic, Adaptational or Treatment-Related?

In regard to the mechanism of altered gene expression in complex human brain disorders, we expect to see three classes of transcriptome changes: *DNA sequence-dependent*, *adaptational*, and *treatment-related*. These are not easy to separate out, but based on the known pathophysiology of the disease, we speculate that they may show somewhat different patterns of expression changes.

In polygenic disorders with an environmental-developmental component such as schizophrenia, DNA sequence-dependent expression changes may not be present in all cases. In a microarray study of schizophrenia, gene expression changes belonging to this class may show 1) a robust, unidirectional change in 50% of comparisons, but unchanged levels in the rest of the comparisons; 2) changes that are present across

multiple brain regions within the same subjects with schizophrenia; 3) uniform changes of transcript abundance across all cells expressing the transcript; and 4) the gene is localized in a cytogenetic region that has been implicated in inheritance of the disease.

On the other hand, adaptational changes would be expected to be present in the majority, if not all subjects with the disease. In schizophrenia, such changes may reflect the combined effect of genetic and environmental insults, and as such, they may be associated with the symptoms of the disease. From a pharmacological standpoint, the common secondary changes may represent much better drug targets than the diverse, potentially subject-specific DNA-sequence dependent changes. Adaptational changes may show a more specific spatial distribution, where the change preferentially affects specific brain regions or subdivisions within a region.

In addition to the regional adaptational changes, we expect that we will find some expression changes due to treatment of schizophrenia. Such changes would also be expected to be common across subjects and to exhibit regional variation that parallels the distribution of the receptors and related neural circuits affected by the medication of interest. For example, due to differential dopamine receptor density within the neocortex one would expect that gene expression changes due to haloperidol exposure would produce changes in dopamine D2 receptor-rich deep layer 5 such as observed by Middleton et al. (72).

## Data Interpretation Issues

Brain tissue is unique by virtue of its complexity, and this complexity must be considered when analyzing the outcome of microarray experiments. Several important topics are discussed below.

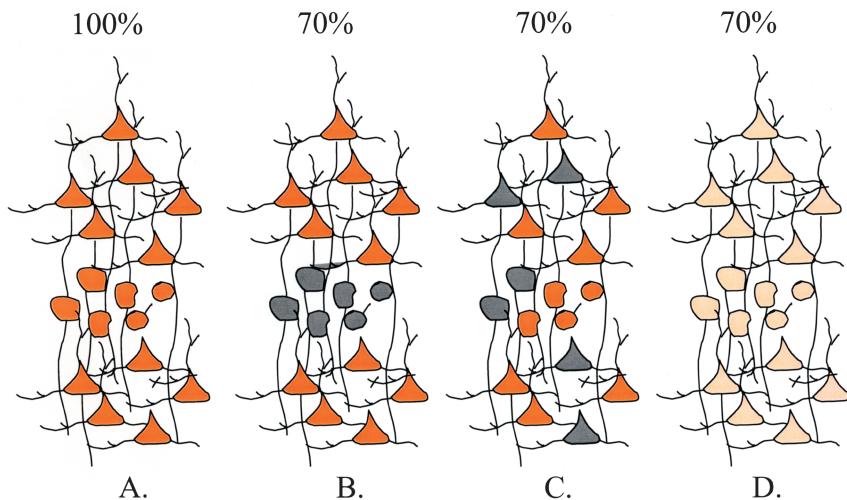
**Detection Limit of Expression.** Biological sensitivity differs from nominal microarray sensitivity. That is, at a sensitivity of 1 in 250,000 copies of mRNA, both cDNA and oligonucleotide microarrays can theoretically detect even the lowest abundance mRNA species in a uniform cell population (40,73,74). However, brain samples usually contain many different cell types, and this phenotypic complexity "dilutes out" sparse mRNAs such that their relative abundance is below microarray detection. For example, approximately 50% of the mRNA abundance in grey matter is comprised of glial cells that dilute the neuronal mRNA signal by half (75). Furthermore, neuronal mRNAs are cross-diluted by transcripts from a diversity of neuronal phenotypes

which do not express the transcript of interest. Consequently, the biological sensitivity may be many fold lower than the nominal microarray sensitivity, and some rare transcripts, easily assessed by *in situ* hybridization (e.g., some neurotransmitter receptors) will not be detected by microarrays. Rare transcripts may be also more susceptible to sample preparation issues, especially if they involve an amplification process (76).

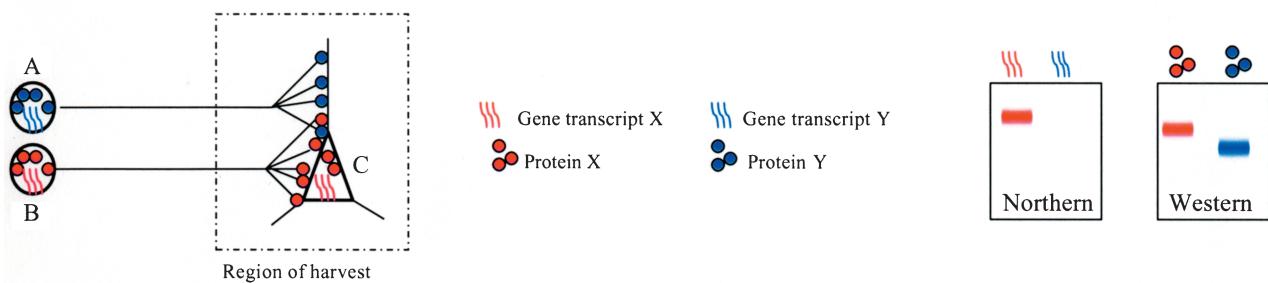
**Fold Change as a Measurement of Differential Expression.** Traditionally, microarray experiments used "fold change" as a descriptor of the magnitude of the measurement difference across samples (45,77). Unfortunately, the reliability of "fold changes" depends on many parameters, including (but not limited to) probe length and properties, absolute abundance of a molecule, data standardization, modeling, tissue, and platform used. Furthermore, a fold change may also be misleading vis-à-vis a biological process of a disease: 20% of neuronal cell loss cannot result in >20% decrease of markers that are expressed in all neurons. For this reason, more statistical approaches (rather than fold cutoffs) may uncover expression changes of modest magnitude that are potentially important for the biological process studied (78).

**Localization of Gene Expression Changes.** Microarrays do not provide anatomical information about gene expression (Fig. 1). A 30% change in transcript may be the result of a uniform transcript downregulation across all cells, a full loss of transcript in a single cortical layer, or diffuse cellular loss. Obviously, this information is critical for data interpretation. Furthermore, expression changes in multiple genes may occur in the same cell type, or may be located in different anatomical compartments (e.g., glia vs. neuron; layer 3 vs. layer 5 neurons). In addition, different family members may perform a redundant function, and they are often co-expressed in cells. As a result, reduced expression or absence of a widely expressed molecule may affect only a small subpopulation of neurons that do not co-express multiple family members. For these reasons, anatomical and co-localization studies will continue to be an integral part of the microarray data interpretation process.

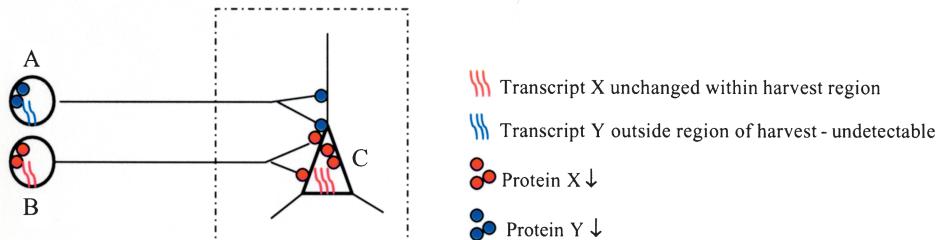
**Transcript Changes vs. Protein Changes.** Although protein levels are often regulated without underlying transcript changes, transcript changes usually result in protein changes, albeit not necessarily to the same magnitude. However, protein sources and transcript sources are often different in the nervous system: mRNA originates predominantly from cell somas in the region of harvest, whereas proteins are also a part of the massive presynaptic neuropil (Fig. 2). Discrepancies



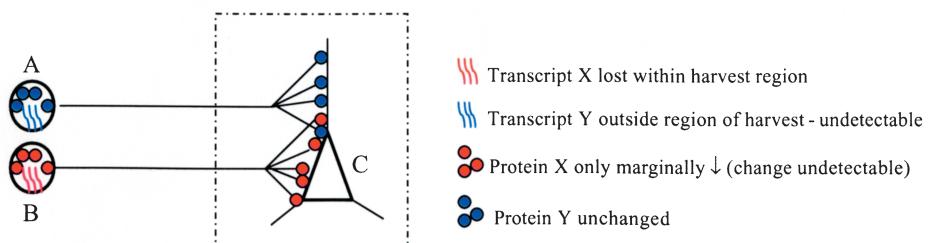
### A. Normal circuitry



### B. Decreased input



### C. Loss of transcript



in protein vs. transcript change at bulk assessment levels (microarrays, RT-PCR, northern hybridization vs. western hybridization) may actually indicate where the change is occurring, and should not be hastily interpreted as “*changed transcript levels did not result in changed protein levels*”.

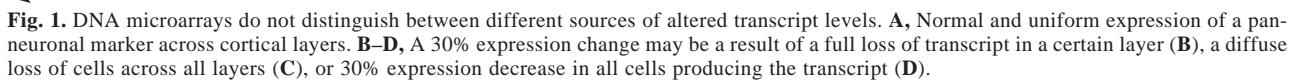
**Comparing Data across Platforms.** Currently, microarrays performed by different groups of investigators are not readily comparable (68–71). Differences in harvesting, isolation procedures, labeling, platform and analysis make comparisons descriptive at the best, permitting only assessments as to whether the pattern of change was found across both experiments. When using standardized microarrays (e.g., Affymetrix Gene Chip) this concern is greatly reduced; however, this standardization may not eliminate all significant sources of unwanted variability (e.g., equipment, operator decisions, batch of label and microarrays, etc.).

### Studying Psychiatric Disorders Using DNA Arrays

Due to the phenotypic complexity and small magnitude of biologically important transcript changes, successful microarray studies of the human brain tissue were performed years after the first microarray experiments were carried out in cancer research. These early studies, primarily focusing on neurological disorders, demonstrated both the power and limitations of microarrays. In analysis of postmortem tissue from a subject with multiple sclerosis (MS), Whitney and coworkers (50,79) identified differentially expressed genes related to immune function, cell cycle and growth, intracellular signaling, adhesion, transport and myelination. A synthetic enzyme for proinflammatory leukotrienes, 5-lipoxygenase, was upregulated in both

brains of MS subjects and mice with experimental allergic encephalomyelitis (EAE), highlighting phenotypic commonalities between an experimental model of MS and the disease itself (79). In elegant studies, Ginsberg and colleagues generated valuable transcriptome profiles from individual senile plaques, tangle-bearing CA1 neurons and basal forebrain cholinergic neurons from Alzheimer’s disease (AD) subjects (80–82). They found differences in gene expression for phosphatases/kinases, cytoskeletal proteins, synaptic proteins, glutamate and dopamine receptors, and cathepsin D in tangle-bearing neurons (81). In contrast, increased abundance of APP, tau, phosphatases, bcl-2, bax, and glutamate receptors (81) were present in the affected CA1 neurons. Furthermore, this group of investigators found that changed expression levels of synaptic proteins and tyrosine kinases in cholinergic neurons of the basal forebrain were highly correlated with mild cognitive impairment of subjects with AD (82). Together, these studies provide evidence that DNA arrays can validate known neuropathologies and are essential in a discovery process that will lead us to “unexpected” findings. For example, expression changes in activity-regulated cytoskeleton-associated protein, focal adhesion kinase, glutaredoxin, and utrophin were associated with neurofibrillary tangle formation (81). These studies facilitated the emergence of the second generation of brain microarray publications that continue generating exciting data in autism (83) and Alzheimer’s disease (84–86) and characterization of animal models in Parkinson’s disease (87–89). However, for all their descriptive power and intriguing data, many studies are falling short in explaining how changed sets of genes interact with each other or whether they are causative or secondary for the brain disorder.

Due to multiple converging factors, the first microarray experiments focused on neurological, and not psy-

**Fig. 1.** DNA microarrays do not distinguish between different sources of altered transcript levels. **A**, Normal and uniform expression of a pan-neuronal marker across cortical layers. **B–D**, A 30% expression change may be a result of a full loss of transcript in a certain layer (**B**), a diffuse loss of cells across all layers (**C**), or 30% expression decrease in all cells producing the transcript (**D**). 

**Fig. 2.** Relationship of transcript changes to protein alterations. The nervous system is unique by the axonal projections that deliver proteins to remote regions (**A**). Changes in these proteins are often independent of local transcript changes and may be a result of transcript changes in cells located at a considerable distance (**B**). Similarly, proteins from distant sources may mask local protein changes in the cellular soma that are a result of local transcript alterations (**C**). Discrepancies between transcript and protein changes can be misinterpreted if the source of the material is not considered in the biological context of the data. In **A**, *protein X* has a dual source of origin, intrinsic and extrinsic, and they can be analyzed by western blots. In contrast, *protein Y* is present from an extrinsic source only. Microarrays and northern hybridization can detect only the intrinsic sources of transcripts—extrinsic transcripts of *genes X* and *Y* are not assessed. In **B**, extrinsic protein sources are noticeably decreased by western blot, but the intrinsic transcript and protein of *gene X* are unchanged. This is not a discrepancy between the findings, but indicative of the biological change occurring in a remote region. In **C**, the intrinsic transcript and protein for *gene X* are lost, and this is discovered by microarrays. However, the extrinsic source of *protein X*, which has a robust presence in the region of the harvest, is unchanged, and masks the intrinsic protein change (but not transcript change). This pattern should be noticed and further analyzed by *in situ* hybridization and immunohistochemistry. Note that in both **B** and **C**, within the same cell, the transcript change resulted in a protein change, yet, at a regional level, this protein change was unmasked.

chiatic disorders. Limited tissue availability, the added complexity of the disorders and the smaller magnitude of expression changes have been challenging issues in obtaining high-quality microarray data for psychiatric disorders. With advancement of microarray sensitivity, improvement in data analysis methods and growing understanding of the strength and limitations of the technology we are expecting a strong emergence of microarray research into psychiatric disorders. In particular, microarray-generated datasets have significantly advanced schizophrenia research and similar breakthroughs can be expected in analysis of major depression and bipolar disease. Furthermore, using animal models, various microarray studies have successfully explored the underpinnings of the behaviors thought to be associated with psychiatric conditions (90–96).

### Schizophrenia as a Disease of the Synapse

Schizophrenia is a multifaceted disorder that affects ~1% of the human population worldwide. The disease fully manifests itself during the post-adolescent period usually with a constellation of psychotic, negative, and cognitive symptoms (97,98). The etiology of the disease is complex, and it involves combined multi-genetic and environmental insults on the developing nervous system (97). The prefrontal cortex (PFC) has been implicated as a major site of brain dysfunction in schizophrenia. Furthermore, in the PFC of subjects with schizophrenia anatomical and immunohistochemical changes have been extensively documented (98).

To investigate if these functional, physiological, and anatomical findings are also associated with alterations of gene expression patterns, we performed a high-density cDNA microarray analysis of PFC area 9 in six pairs of subjects with schizophrenia and matched controls (99). Our initial microarray data uncovered prominent changes in the expression of genes related to presynaptic secretory release. We also observed robust changes in genes related to GABAergic and glutaminergic transmission, which may be secondary to the altered presynaptic drive. Importantly, the presynaptic expression deficits observed in schizophrenia appeared to be subject-specific. That is, although all subjects showed decreased expression of multiple presynaptic gene products, the specific combination of affected transcripts varied from subject to subject. This phenotypic diversity may argue for a continuum of molecular phenotypes in schizophrenia, perhaps similar to the variability in the clinical manifestations of the disease between subjects. In addition to this gen-

eral molecular diversity seen across subjects, we also observed consistent changes in N-ethylmaleimide-sensitive factor and synapsin 2 expression in the majority of subjects with schizophrenia.

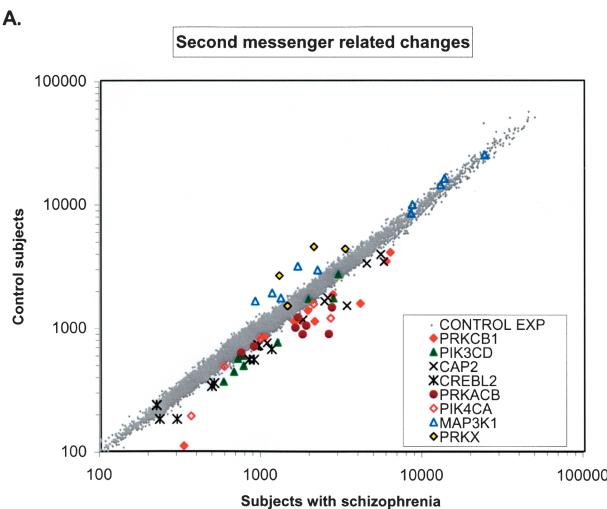
In a similar type of pathway analysis of the same subjects with schizophrenia, we found that genes grouped by metabolic pathways revealed consistent changes in the ornithine-polyamine metabolism, mitochondrial malate shuttle system, transcarboxylic acid cycle, aspartate and alanine metabolism and ubiquitin metabolism (72). Verification of microarray data by *in situ* hybridization also confirmed altered expression of soluble malate dehydrogenase, mitochondrial glutamate-oxaloacetate transaminase type 2, ornithine decarboxylase antizyme inhibitor and ornithine aminotransferase. These findings suggest that altered metabolism may be an important component of the disease process in schizophrenia.

The same microarray data set also revealed multiple changes at the postsynaptic cell. These, amongst others, involved AMPA2, a G-protein-coupled receptor (GPCR) and regulator of G-protein signaling 4 (RGS4; 100). The decreased RGS4 expression in subjects with schizophrenia may have a profound effect of GPCR-mediated signaling in general; this family of proteins modulates signaling through the GPCRs and is expressed in a cell-specific anatomical distribution (101–104). More specifically, RGS proteins are GTPase activating proteins that facilitate G $\alpha$ -bound GTP hydrolysis and thus limit the duration and timing of the GPCR signal (105,106). Hence, downregulation of RGS4 in the PFC may compensate for decreased synaptic drive by increasing the duration of signaling from GPCR activation. In addition to adaptational changes, RGS4 may also represent a schizophrenia susceptibility gene as indicated by the increased transmission of allelic variations in the 5' region in subjects with schizophrenia (107). However, it remains to be determined if RGS4 is the sole susceptibility gene on chromosomal locus 1q21–22 (108).

The functionality and biological effects of the decrease in RGS4 transcript are not known. However, the interaction of the RGS family with multiple G-alpha subunits raises the possibility that changed RGS4 expression results in multiple adaptational transcript alterations related to second messenger systems. Although the data did not meet our stringent signal/background microarray criteria, we observed a highly correlated expression change between RGS4 levels on one hand and three G-alpha subunits (G-olf, Gq, and Gil) on the other (data not shown). In addition, our microarray findings seem to implicate multiple genes involved in both

phosphatidylinositol and cAMP signaling pathways (Fig. 3). These general findings have been reported in the past by others with various consistencies, and they likely represent adaptational changes that are functional consequences of more specific upstream changes in GPCR signaling, possibly involving the dopamine signaling systems (109–112). In contrast, in a study of a chronic haloperidol-treated and matched control monkey the same microarray analysis did not detect expression changes in the second messenger systems, suggesting that our findings may not be related to antipsychotic treatment. However, follow-up studies will have to determine if these expression changes co-localize in a well-defined cell sub-phenotype or affect diverse cell populations.

The altered expression of presynaptic, postsynaptic and energy metabolism genes in our dataset are likely to be interrelated, although the causality of the

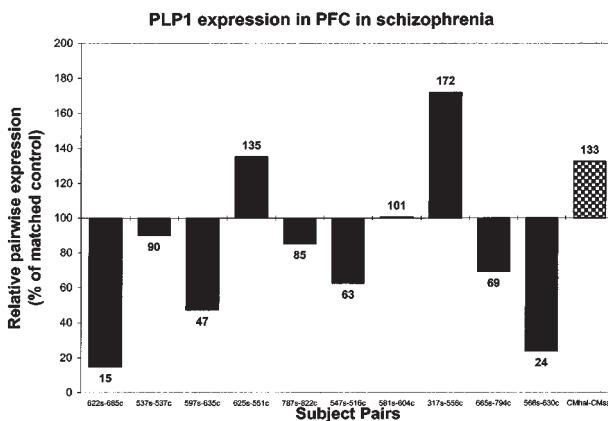


**Fig. 3.** Expression changes in second messenger genes in subjects with schizophrenia. Changes in GPCR signaling may involve a combination of genetic and adaptation changes. Our data is suggestive of an adaptational expression change both in the phosphatidylinositol and cAMP signaling pathways. The graph depicts the relative expression of 10 pairs of schizophrenic subjects and matched controls for 6 genes (PRKCB1, protein kinase C, beta 1; PRKACB, protein kinase, cAMP-dependent, catalytic, beta; PIK3CD, phosphoinositide-3-kinase, catalytic, delta; PIK4CA, phosphatidylinositol 4-kinase, catalytic, alpha polypeptide; PRKX, protein kinase, X-linked; CAP2, adenylyl cyclase-associated protein 2; MAP3K1, mitogen-activated protein kinase kinase kinase 1). Each symbol represents the relative expression in one comparison. Due to different microarray batches, not all genes were detected on all 10 microarrays. X axis depicts microarray reported expression levels in schizophrenia subjects, Y axis reports expression levels in control subjects. The cloud of small gray dots shows the distribution of >8,000 genes in one same-sample hybridization experiment and corresponds to assay noise. Note that most of the depicted second messenger genes showed a change in schizophrenic subjects that was outside the range of the established noise.

changes is unclear at the present time (46,72). Decreased or inefficient synaptic drive could lead to secondary down-regulation of energy metabolism and altered signaling at the postsynaptic membrane. Alternatively, decreased metabolism may result in decreased synaptic drive and increased pruning, which would in turn lead to postsynaptic expression changes. Finally, intracellular signaling deficits may lead to decreased energy metabolism and increased synaptic pruning. However, processes involving all three systems cannot be excluded at the present time.

Other investigators, using different cohorts of subjects, also observed some of our microarray findings. Sklar and co-workers reported that NSF was decreased in the PFC of schizophrenic subjects in a different cohort (113), and changed expression of genes related to polyamine metabolism were reported by Jansen researchers using Agilent cDNA arrays (114). Vawter and co-workers, using membrane arrays, also observed expression changes of genes related to ubiquination, presynaptic secretion, glutamate system and malate shuttle (115,116). In addition, Feron et al. reported decreased RGS4 expression in fibroblasts harvested from patients with schizophrenia (117), while Hembly et al., in analyzing single cells from subjects with schizophrenia, found that the expression levels of several synaptic markers were decreased (118). Bahn and co-workers found that changes in ApoL1 and ApoL2 expression are characteristic of several independent cohorts of subjects with schizophrenia. Although we did not find ApoL expression changes in our cohort of subjects, differences in microarray probe sets may account for this (66).

Some other aspects of expression changes in schizophrenia are also important to point out. Hakak et al. (119), using Affymetrix GeneChip reported a defect of myelination that may be due to altered oligodendrocyte development. We report here a changed expression of proteolipid protein 1 (PLP1) across the majority of subjects with schizophrenia (Fig. 4). This PLP1 expression decrease does not appear to be related to antipsychotic medication: in a microarray comparison of a pair of haloperidol and saline-treated monkeys (120,100) we did not observe the same expression deficit. Schneider et al. (121,122) suggested that PLP1 has a dual function, active in glial cell development, and a distinct role in myelin assembly. A block in oligodendrocyte maturation is evident in "jimpy" mutant mice in which mutation of PLP1 leads to a defect in normal transcript splicing (122,123). Furthermore, Schneider et al. (121) showed that this misfolded PLP seen in jimpy is by itself the primary cause of abnormal



**Fig. 4.** PLP1 expression is decreased across the majority of subjects with schizophrenia. PLP1 cDNA microarray expression data from 10 subjects with schizophrenia (s) and matched controls (c). Last column represents a matched pair of chronic haloperidol-treated (CMhal) and saline-treated (CMsal) monkey. Subject pairs are denoted on X axis, expression levels are denoted on Y axis as percent of the matched control. Note that the majority of subjects exhibit a decrease in PLP1 expression. This reduced expression was not present in the chronic haloperidol-treated monkey.

oligodendrocyte death. Finally, it is interesting that the absence of PLP produces more devastating effects than those of dysmyelination (125). Given these converging findings, we propose that the PLP1 expression decrease may be a significant contributor to decreased expression of oligodendrocyte development in the PFC in schizophrenia (119). It is not clear how these data will fit into a comprehensive biological model of schizophrenia, but it seems to suggest that there is both a neuronal and glial involvement in the disease.

### The Future: Integration of Biological Knowledge

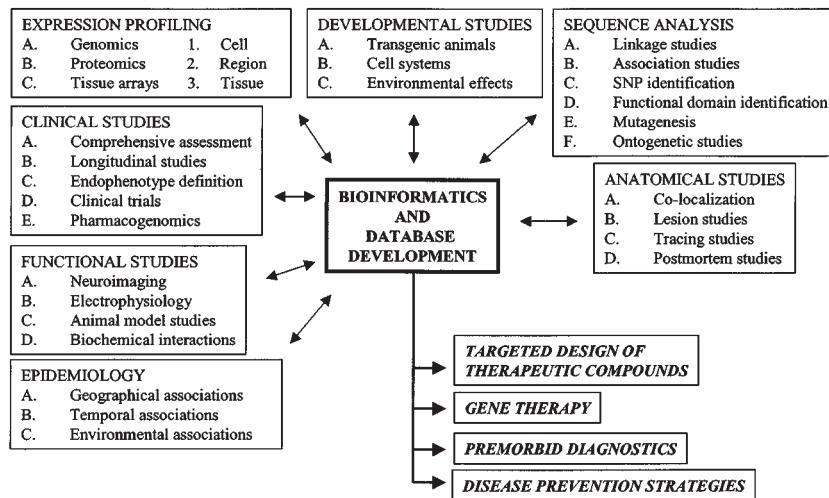
In the near future, critical biological data will be generated on multiple fronts:

1. *Single cells of defined phenotypes from the diseased brain will be analyzed for complex gene expression patterns in a high-throughput fashion.* Using laser-capture dissection of identified single cells, it is now possible to pool members of a subclass of cells and analyze them using high-throughput genomic and proteomic techniques (80–82,126–128). The characterization of gene expression patterns in neuronal populations defined by a shared axonal projection target and/or electrophysiological properties will advance our understanding of the specific neu-

ronal phenotypes that participate in the disease process.

2. *Gene expression patterns or genetic vulnerability may predict symptoms and drug responsiveness at the individual patient level.* Functional clinical studies will define the significance of gene expression alterations related to the psychiatric disorder and further clarify clinical diagnosis. Egan et al. (129) demonstrate the significance of a common polymorphism for COMT in schizophrenia and highlight the combined power of neuroimaging and genetic studies.
3. *DNA sequence information and bioinformatics will define co-regulated gene networks.* Expression changes will be modeled based on common DNA sequence characteristics, and frequency and positional cloning will be combined with prediction of transcription factor binding sites (130,131). Co-regulatory networks will be defined using expression and sequence information. By systematically parcelling the whole brain into small cubes (“voxels”) and identifying their “3D transcriptome” one can get an in-depth view of how gene expression is co-regulated across brain regions (132).
4. *Novel animal models of disease endophenotypes and traits will be developed.* Multiple transgenic animals, with different gene alterations, may model a variety of disease aspects. These studies will greatly complement postmortem studies, and will be driven by the combined knowledge of many other fields, including functional, inheritance, and transcriptome studies relating anatomical expression data to the syndromal disease under investigation.

Obviously, the integration of the data from different sources is a monumental task, and it has to become a priority. In sharing data sets it has become imperative to develop common protocols and determine the minimal information that must be included (61). The Microarray Gene Expression Group (MGED) is a working group of scientists that have drafted standards for protocols and language that address some of these concerns in microarray research (133). To aid the meaningful comparison and linking of datasets, the *minimal information about a microarray experiment* (MIAME; 134,135) will likely become a gold standard for microarray data deposition into the Gene Expression Omnibus (GEO; 136), ArrayExpress and GeneX databases



**Fig. 5.** The future is in sharing knowledge across multiple fields of research. Better integration of data will take advantage of new knowledge bases. In addition to genomic and proteomic studies, the characterization of gene expression patterns in neuronal populations defined by a shared axonal projection target and/or electrophysiological properties will advance our understanding of the specific neuronal phenotypes that participate in the disease process. Furthermore, steady improvements in neuroimaging techniques will allow functional imaging studies to define the type and location of disturbances related to psychiatric disorders; novel transgenic animal models of disease endophenotypes and traits will be developed; linkage studies and DNA screening will identify most of the susceptibility genes for multigenic psychiatric disorders; the effects of environmental insults on the developing brain will be defined. Finally, clinical data will be correlated with research findings. The combined information will greatly facilitate the development of novel therapeutic approaches and prevention strategies.

([http://www.ncgr.org/research/genex/other\\_tools.html](http://www.ncgr.org/research/genex/other_tools.html)) that have already adopted the draft recommendations of the MGED. These databases offer the promise of the new modes of data integration that may facilitate the design of lead compounds for disease treatment and perhaps prevention in a more targeted and hypothesis-driven fashion.

Much work remains to be done. In the future, advances in multiple fields will impact our understanding of the biological basis of psychiatric disorders (Fig. 5). Integration of data across technologies will allow the efficient use of relevant knowledge from diverse sources, and this will take research strategies to a new level.

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