Functional genomics approaches to understanding brain disorders

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The completed draft of the human genome sequence has facilitated a revolution in neuroscience research. This sequence information and the development of new technologies used to analyze gene expression on a genomic scale provides a new and powerful means to investigate brain disorders of unknown etiology and to isolate novel drug targets for these disorders. The term functional genomics broadly describes a set of technologies and strategies directed at the problem of determining the function of genes, and understanding how the genome works together to generate whole patterns of biological function. The most powerful of these functional genomics approaches, expression profiling or DNA microarrays, can be used to analyze the expression of thousands of genes simultaneously. The results to date from the application of DNA microarray methods to postmortem diseased human brain tissue, animal models and cell culture models of brain disorders provide an exciting glimpse into the future of this field.

Keywords: animal models, brain disorders, cDNA arrays, cell culture, chemical mutagenesis, data mining, data sharing, DNA microarrays, gene expression profiling, *in situ* hybridization, mRNA, postmortem, proteomics, oligonucleotide arrays, single nucleotide polymorphisms, therapeutics, transgenics

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As with every other area of biomedical research, the completed draft of the human genome sequence has provided tools to dramatically accelerate neuroscience research. The Human Genome Project has led to the creation of databases containing vast amounts of gene sequence data and the isolation of thousands of partial cDNA sequences representing previously undiscovered genes [1,2]. This sequence information and the development of new technologies to analyze gene expression have now made it possible to analyze gene expression on a genomic scale providing new and powerful means to investigate brain disorders of unknown etiology and to isolate novel drug targets for these disorders. The vast sequence database that is now available will enable investigations aimed at the determination of the function of these known genes within the context of neural systems. Alterations in regional and/or global gene expression patterns can point to biochemical pathways and regulatory mechanisms underlying a disease state. These data will also uncover thousands of new potential targets for drug discovery.

The next era of genomics involves elucidating the function of these thousands of newly discovered genes. In this respect, functional genomics describes studies directed at this process of determining the function of genes and how the genome works together to generate whole patterns of biological function [3]. These methodologies serve to complement the traditional techniques that are limited to the study of several genes at a time (e.g., Northern blotting, RNase protection, reverse transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization) and include DNA microarrays, differential display PCR [4], serial analysis of gene expression (SAGE) [5], total gene expression analysis (TOGA) [6] and mouse chemical mutagenesis [7].

DNA microarrays are the most powerful of the techniques that address global functional genomics questions. They allow for the expression analysis of thousands of genes in a single experiment, thus facilitating the study of global gene patterns that are affected in symphony by specific diseases and drug treatments [3,8]. Therefore, in this review we will focus our discussion on the use of DNA microarrays to understand the biological function of the genome and how these investigations could be of particular relevance to the understanding of brain disorders and the identification of drug targets of relevance to these diseases. We will also consider similar applications of proteomics, the study of the complete set of expressed proteins.

The brain is the most complex organ in the human body and is one of the organs with the greatest diversity of gene expression. The recent completion of the draft of the human genome sequence has resulted in the total number of genes being revised downward to between 30,000–40,000 [1,2] and the expression of thousands of these genes is exclusive to or highly enriched in the brain [9]. The brain regulates complex tasks involved in cognition, emotion, memory, integration of sensory information and motor coordination [10]. Disturbances in these

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functions underlie many brain disorders. Such complex tasks mediated by the CNS draw on multiple brain regions and biochemical systems. To understand the alterations in genetic programs that result in altered gene expression and protein function it is necessary to utilize technologies that can address multiple changes in gene expression in various brain regions, simultaneously. In this regard, DNA microarrays have been used to investigate the regional differential expression of neural genes in mouse and human genomes [11,12]. Furthermore, the use of functional genomics approaches could lead to novel methods of disease diagnosis. Gene expression patterns that are associated with a specific disease could potentially be used as diagnostic markers for the disease. This type of analysis would be especially useful for the diagnosis of psychiatric disorders such as schizophrenia and bipolar disorder, that do not have biological markers and are diagnosed based on behavioral phenotype [13].

DNA microarrays

There are two major types of DNA microarrays, oligonucleotide arrays and cDNA-based arrays. Each of these array types shares some basic common procedures. They both utilize nucleic acid probes bound to a solid surface and hybridization of mRNA species from tissue or cell culture to these probes followed by detection and quantification of these bound molecules to determine differences in mRNA expressions under different conditions, e.g., disease or drug perturbation. **Table 1** displays a comparison of the advantages and disadvantages of these two types of DNA microarrays. In addition, we will also discuss membrane-based macroarrays in detail later in the article as an alternative.

Oligonucleotide arrays

One microarray method involves the massively parallel synthesis of oligonucleotides onto chips. Though several such technologies exist, this approach is perhaps best exemplified by Affymetrix GeneChips™ (Santa Clara, CA). These GeneChip arrays can be used to investigate thousands of genes, simultaneously. The Affymetrix GeneChip technology uses high-density arrays of oligonucleotide probes to efficiently screen large quantities of genetic information. Affymetrix uses light-directed chemical synthesis combining photolithography and solid-phase chemistry to manufacture these probe arrays as described in detail elsewhere [14,15]. Oligonucleotide arrays use 16–20 perfect match/mismatch probe pairs to establish expression levels for each transcript analyzed. This strategy enhances the specificity and reproducibility of GeneChip experiments by averaging quantitative results across multiple probes per gene. In this way potential errors due to array defects, background noise, sequencing errors, DNA polymorphisms, cross-hybridization to similar sequences or ineffective probes are reduced [14,16]. This probe pairing array strategy is one of the major advantages of oligonucleotide arrays. Other advantages of this array type are that the resulting data are absolute and readily cross-comparable across arrays. One of the major disadvantages is that these arrays contain a defined set of transcripts that cannot be easily changed. The research is therefore limited to the arrays available for commercial use. Affymetrix does offer custom arrays. However, the cost is prohibitive for most academic researchers.

cDNA arrays or robotically spotted arrays

In contrast to oligonucleotide arrays, cDNA or spotted arrays are made by baking or using UV irradiation to couple DNA fragments, e.g., PCR products or cDNA clones, to a glass surface [17,18]. Genes are represented by single full-length cDNAs or DNA fragments that are usually several hundred base pairs in length. Typically, probes are prepared from mRNA from two different tissue sources, each labeled with a different fluorescent tag. The probes are hybridized to the arrays and the signal from each fluorescent probe is quantified. The ratio of the signal from the two colors is then used to detect differences in expression between the two tissue samples. The use of such a ratio provides an internal control that reduces experimental variance and improves the sensitivity to differences in message levels. Excellent in-depth descriptions of cDNA array methodology have previously been described [19-21].

The advantages of cDNA arrays are:

- they are relatively inexpensive after initial set-up
- they have great flexibility in the design of custom arrays
- the sequences of target DNAs do not have to be known

cDNA arrays are usually not as sensitive or as reproducible as the Affymetrix GeneChip arrays. However, Yue *et al.* [22], have reported cDNA arrays to be a highly sensitive and reproducible methodology. cDNA arrays are also less effective at distinguishing members of multi-gene families or splice variants of the same gene. The equipment used to robotically spot and read arrays can be

Table 1. Comparison of cDNA *versus* oligonucleotide microarrays.

Advantages	Disadvantages
Oligonucleotide arrays	
Simultaneously measure thousands of genes	Not very flexible
Relative lower initial set-up cost	Expensive chips and ongoing costs
High specificity and reproducibility	
Effective at distinguishing members of gene families with high sequence identity	
Can detect splice variants	
Data are absolute and cross-comparable across arrays	
cDNA arrays	
Simultaneously measures thousands of genes	Lower sensitivity
Relatively inexpensive after initial set-up	High initial set-up cost due to cDNA libraries
Flexible – easier to customize	Greater likelihood of detecting false-positives
Controls and experimental subjects can be compared on the same microarray	Less able to distinguish members of gene families

obtained and set-up in-house at modest expense. However, obtaining and managing the clone libraries can be costly. Many companies also manufacture DNA arrays and the different reagents for their use. A list of companies and their array applications has previously been reported [23,24].

Membrane-based macroarrays

Membrane-based macroarrays can be used as a very sensitive, inexpensive and reproducible alternative when a smaller number of genes need to be analyzed [25]. In this case, DNA is spotted onto relatively large nylon membranes and probes are either labeled radioactively (^{33}P) or with florescent dye. Radioactively labeled probes are the most sensitive and allow for the use of minimal amounts of sample. In contrast to glass DNA arrays or DNA chips they can be hybridized multiple times after probe removal.

The detection of SNPs

Another powerful application of microarray technology is the massively parallel detection of single nucleotide polymorphisms (SNPs). This technology does not directly measure gene expression and it may be debatable whether it belongs under the rubric of functional genomics. However, it does address the role of genomic variation in the function of genes and in particular may greatly accelerate association studies in human populations. In the broader sense, SNP detection will in turn contribute to the understanding of gene function and the role of variation in genomic function.

In an association study, the possible role of a genomic variant in a disease or trait is tested by comparing allele frequencies between groups of unrelated individuals. Such a distortion in allele frequencies can result if the genetic variant plays a role in the predisposition to the illness, or if it is extremely close to such a functional variant (linkage disequilibrium). Association studies have several advantages over linkage approaches to mapping disease loci including a much finer genomic resolution and a greater sensitivity to small gene effects. However, this finer genomic resolution has made genome-wide application of this approach unfeasible until recently because of the large number of markers necessary [26,27]. To achieve the average 50 kb spacing that is likely to be necessary approximately 60,000 markers will be required. SNPs make ideal markers for such applications because of their abundance, ease of detection and lower mutation rate. Recently, accompanying the completion of the human genomic sequence, millions of SNPs have been identified that are suitable for such studies [2,28]. A variety of microarray-based methods for SNP detection have been proposed but most involve PCR amplification of DNA flanking the SNP, a single base extension reaction in order to differentially label the variant base with a fluorescent tag and then hybridization of thousands of SNPs on a microarray chip for parallel detection.

Applications may involve not only mapping of disease susceptibility genes but also genes involved in drug response.

Proteomics

The analysis of global changes in protein expression complements functional genomic approaches aimed at profiling RNA alterations. Analysis of protein expression is important because the final expression product rather than an intermediate is measured, providing a more direct analysis of changes in protein concentrations. In addition, proteomics can address changes that RNA analysis alone cannot, including protein-protein interactions, post-translational modifications and protein abundance [29]. Proteomics aims to identify all human proteins and their function, thus it is more nascent than functional genomics. Proteomics is more of a challenge, since approximately 30,000–40,000 human genes would give rise to millions of chemically distinct proteins after various post-translation modifications [30]. Therefore, unlike genomics, currently proteomics can at best address a subset of possible proteins. In this case, global analysis of proteins involved in disease and drug targets would be of high priority.

The most common approach that has been used to determine which proteins are expressed in a tissue of interest is two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). Typically, proteins from a tissue of interest are electrophoretically separated, first in one dimension by charge. Then SDS is added and the samples are separated in the second dimension by molecular weight. 2D PAGE technology has been in use for several decades. This method was first used in the mid-1970s around 20 years before the invention of DNA microarrays [31-33] and has been employed in a variety of applications to investigate the mechanisms underlying brain disorders. For example, to investigate the causes of Creutzfeld-Jacob disease (CJD) where two members of a protein family have been identified in the cerebral spinal fluid of CJD patients that differentiate CJD patients from patients suffering from other dementias [34].

This technology has undergone a recent renaissance due to the development of technologies to scan 2D gels using mass spectrometry. Several thousand spots on a 2D gel, representing one or more proteins, can be analyzed in an automated fashion by mass spectrophotometry [35-37] to determine the most probable amino acid sequence. Translated DNA databases can then be used to determine the identity of each protein [30]. Once these proteins are identified their expression levels can then be monitored in the brain disease of interest. Unfortunately, 2D PAGE gels can currently only be used to resolve high abundance, soluble, cytoplasmic proteins [38]. In addition, this technology has difficulty detecting large, low abundance, hydrophobic or basic proteins and has current limitations in the number of proteins that can be resolved [29,35].

Proteomic methods are currently limited in the number of proteins that can be interrogated and therefore, the data sets produced from protein analysis are much smaller than those that result from RNA expression profiling. To make proteomics a more viable approach to the investigation of global protein changes in brain disorders, many of the limitations of this technology need to be addressed. Future advances in this technology are described in Celis *et al.* [29], and include improvements in quantification methods, high-throughput analysis and systems to support 2D gel comparisons.

The Trinectin™ Proteome Chip, which was recently announced by Phylos, will be one of the first high-throughput methods using protein arrays and is an example of the coming wave of new proteomic technologies. The major strength of this new technology is the ability to automate the analyses of the thousands of proteins attached to each chip. The Trinectin Proteome Chip will allow investigators to analyze the relative protein expression profile of thousands of proteins, analogous to the capabilities of DNA microarrays, that are affected by a specific brain disorder or drug [101]. The combination of DNA microarrays and Trinectin Proteome Chips (and other similar technologies) will provide a very powerful means to address the etiology of brain disorders.

Data mining

High-throughput technologies used to measure gene expression e.g., DNA microarrays, yield vast quantities of data. One of the most daunting challenges of these global gene expression studies is making biological sense out of this vast quantity of data. The identity of genes whose expression is changed can be determined through BLAST [39,40] searches of Genbank™ and other databases comprising both sequence and functional data. The function of unknown genes can be inferred by homology both within and across species. A variety of software tools have been developed to aid in the interpretation, organiza-

tion and visualization of large-scale gene expression profiles. Tables 2 and 3 display a list of software and databases, respectively, that facilitate the analysis of microarray data.

Data analysis tools have recently been developed that are changing the way the massive gene expression data sets are analyzed [41]. These strategies facilitate the isolation of significant details contained in microarray data and include hierarchical cluster analysis [42], self-organizing maps [43,44], support vector machines [45], k-means clustering [46], principal components analysis [47] harvesting of expression trees [48], gene shaving [49] and gene group analysis [50].

Hierarchical cluster analysis is one of the most widely used of these tools. It identifies groups of genes that are co-regulated, to reveal information about possible functional and physical connections. There is often a strong similarity in function within clusters of co-expressed genes. However, hierarchical cluster analysis can cluster randomly generated data points or unrelated transcripts [51]. Clusters of co-expressed genes that are associated with common function could potentially facilitate the discovery of common trans-regulators e.g., an upstream protein significantly involved in the etiology of a specific brain disorder. Based on expression data, yet uncharacterized genes that are part of a gene co-regulation network could suggest biological function [52] and common patterns of gene expression could provide insight into biochemical pathways and regulatory mechanisms that could be of relevance to the etiology of specific brain disorders.

Similar to functional genomics, proteomics faces the challenge of analyzing large amounts of data. In this respect, various data mining tools have also been applied to proteomics data. Some of these tools include heuristic clustering [53], similarity clustering [54] and regulatory homology [35].

Data sharing

Sharing DNA microarray data would be advantageous and is supported by many researchers in the field. In this regard, sharing these data could improve analysis methods, enhance confidence in results and facilitate comparisons between experiments [55]. However, several issues relevant to quality, format and validation need to be addressed before microarray databases would be meaningful [56]. In addition, investigators who use shared data need to be aware of the limitations of this technology. Without this knowledge, even high quality validated microarray data could result in confusing secondary analyses [51]. These issues, proposals for public microarray databases [51,55,57] and websites currently used for microarray data sharing [56] have been previously addressed in detail.

Gene profiling: limitations

General technical limitations

The failure to detect gene expression

The sensitivity of microarray methods may not be adequate to detect low abundance transcripts that nevertheless play a critical functional role. This may be particularly true in brain [58].

The failure to detect gene expression differences (false-negatives)

A single microarray study might detect gene expression changes at the 1.5-fold level. However, functionally significant expression changes could occur below this level of detection resulting in false negative results [58].

The detection of false-positives due to variability in experimental factors

This limitation can be addressed by performing multiple replicates of each experiment, as well as replicates of each chip with the same mRNA sample [59]. As this rapidly becomes cost prohibitive, a pressing question is how many chips are necessary to achieve the required level of statistical certainty. As the variance is both biological and experimental in nature, this number likely depends on numerous biological and experimental variables that are specific to the tissue and the experiment. Pilot experiments are usually conducted to empirically determine the nature of the variance and the number of replicates required. Other traditional methods of mRNA detection such as RT-PCR or *in situ* hybridization must be used to validate changes in gene expression. In the case where a small number of gene changes are identified, *in situ* hybridization is preferable since this method also provides important anatomical localization data [51].

Assigning function

Only a portion of mammalian genes have been isolated and associated with function. Function can be inferred for a larger portion of genes based on homology. As of this writing, most array technologies interrogate only a subset of all genes and the function is known for only a portion of those. Therefore, a comprehensive analysis of global gene function is not yet possible and functional conclusions must be limited.

Table 2. Data visualization and analysis resources.[1]

Software	Source	Website
GeneSpring	Silicon Genetics	www.sigenetics.com
DecisionSite	Spotfire	www.ivee.com
Cluster	Michael Eisen	rana.stanford.edu/software/
Tree View	Stanford	
Stanford's ScanAlyze	Eisen Lab Stanford	http://rana.lbl.gov/EisenSoftware.html
MicroArray Explorer	Peter Lemkin NCI/FCDRDC	www.lecb.ncifcrf.gov/mae/
MineSet	Silicon Graphics	www.sgi.com/software/mineset/
GeneSight	Biodiscovery Inc.	www.biodiscovery.com
ImageQuant	Molecular Dynamics	www.mdyn.com
IPLab	Scanalytics	www.scanalytics.com
ArraySuite	NHGRI	www.nhgri.nih.gov/DIR/Microarray/ image_analysis.html
GenePix	Axon Instruments	www.axon.com
Microarray Suite	Affymetrix	www.affymetrix.com
Stanford Microarray Database	Stanford	genome-www4.stanford.edu/MicroArray/ SMD/
Rosetta's Resolver	Rosetta Biosoftware	http://www.rosettabio.com/products/ resolver/default.html
TIGR'S Array Viewer	The Institute for Genomic Research	http://www.tigr.org/softlab/
EBI's Expression Profiler	European Bioinformatics Institute	http://ep.ebi.ac.uk/
J-Express	Bioexchange	http://www.bioexchange.com/tools/ softwaredetail.cfm?SID = 40
Lists of software and other analysis tool web links	Leming Shi's DNA microarray site	www.gene-chips.com

[1]*Adapted from [93,94].*

Changes in mRNA levels may not reflect changes at the protein level

The transcription of genomic DNA into mRNA is a necessary step for successful protein synthesis, and changes in gene expression can lead to phenotypic and morphological changes that are the result of various environmental perturbations and stressors [3] and are therefore indicative of cell state and gene activity.

Post-translational protein modifications

A disease process could be the result of post-translational protein modifications, which are independent of changes in mRNA expression.

If the expression of genes that are primary to the etiology of a brain disease are not altered they will not be detected by microarray analysis

A disease gene may contain a mutation that leads to the dysfunction of the resulting protein but does not affect the level of its mRNA. Many diseases such as X-linked adrenoleukodystrophy [60] are caused by gene mutations that do not result in alterations in the expression levels of that gene and therefore would not be detected by DNA microarray analysis. Apparently one of the main benefits of DNA microarray technology may be identifying the secondary consequences of the primary underlying disease effects on gene expression. If these gene expression changes are a consequence of the disease state they could contribute to the long-term nature of a brain disorder as well as the inability of certain patients to remit.

Limitations of particular relevance to brain disorders

Diversity of brain regions

Brain regions contain a tremendous diversity of cellular subtypes and gene profiling of entire brain regions or subregions cannot address small changes in gene expression in genes specific to a small number of cell types [19,20,58]. This limitation becomes even more significant for genes

Table 3. Sequence databases.		
Database	**Source**	**Website**
Genbank	NCBI	www.ncbi.nlm.nih
Ensembl	Sanger Centre	www.ensembl.org
Golden Path	UCSC	genome.ucsc.edu
Celera Discovery System	Celera	www.celera.com
LifeSeq Gold	Incyte Genomics	http://www.incyte.com/

expressed in low abundance. However, microdissection techniques that can address gene expression patterns in specific cell types and subregions have recently been developed. For example, Luo *et al.* [61] have described a technique called 'Laser Capture Microdissection' to perform microarray analysis on individual neurons. In addition, *in situ* hybridization of mRNAs of interest can be used to further examine differential gene expression in specific cell types and regions.

Brain tissue usage

The use of brain tissue introduces variables such as dissection methods, tissue preservation and postmortem interval (discussed in the section on postmortem studies) contribute to variation making it more difficult to reproduce results [19,20].

Changes in gene expression

If changes in gene expression relevant to the cause of a specific brain disorder are restricted to a specific time period in development, then it is unlikely that such a genetic etiology could be detected by studies of adult postmortem tissue or in studies of adult animals.

Heterogeneity of gene expression between people

Controls and patients could make it difficult to differentiate between inter-individual differences in gene expression and those changes relevant to the disease process [19]. Heterogeneity of the disease process at the molecular level could also make it very challenging to detect similar expression pattern changes among patients that have the same brain disorder [10,58]. For example, schizophrenia includes a wide range of clinical phenotypes that most probably are also caused by a wide range of molecular heterogeneous changes. One way to address this limitation is to use large group sizes to increase the probability of identifying subgroups of overlapping gene expression changes in the patient population. However, the sample sizes necessary to detect small gene effects may make such studies impractical.

Application of gene expression profiling to understanding brain disorders

In the next section we will describe the applications of gene expression profiling, which include postmortem studies and animal and cell culture models of brain disorders.

Postmortem studies

The mechanisms underlying the pathophysiology of many human brain disorders remain unknown. Genetic material that is preserved in postmortem human brain tissue provides a powerful means to investigate some of these disorders. However, there are some significant limitations for the use of postmortem tissue to understand brain disorders. Barton *et al.* [62], evaluated the influence of postmortem interval, time between death and RNA extraction from brain, on RNA degradation. The postmortem interval has only a small effect when tested within 36 h of death and most probably does not account for the wide variation detected in human brain RNA levels. Apparently there is not a large degree of RNA degradation in the first 36 h after death. However, longer intervals and variability of handling procedures e.g., tissue freezing and storage could lead to RNA degradation. Johnston *et al.* [63], have reported that mRNA levels in human brain decrease as much as 200% after a 48 h postmortem interval and this degradation could contribute to the wide individual variation in RNA levels.

In addition, other potentially confounding factors include the patient's condition just prior to death [62] and correct diagnosis. For example, if the patient's death is caused by a condition other than the brain disorder of interest, e.g., hypoxia or some other complicating condition, mRNA levels could then be influenced by these additional factors. Moreover, most patient populations are under drug treatment, which provides an additional, confound to RNA

expression data. Many drugs have been shown to have significant effects on mRNA expression in rodent brain [64-66]. For example, many studies have been performed aimed at evaluating RNA changes in the brains of schizophrenic patients. All these studies have used postmortem tissue from patients and controls and a majority of these patients have taken antipsychotic drugs. These drugs have been shown to influence the expression of various genes in rat and primate models [64,67]. Therefore, it is often not clear if RNA changes detected in the brains from individuals that have schizophrenia have relevance to the disorder or the drug treatment. In addition, many patient populations experience many other factors (due to their illness) that are different from the control population such as diet, lifestyle etc. that could also contribute to RNA changes. It also is not clear if RNA changes detected in postmortem brain tissue are primary to the disease or secondary effects that result from the primary causes of these diseases.

Applications of gene expression profiling to postmortem tissue of diseased human brain

DNA microarrays have been applied to gene expression profile analysis of diseased and control brain tissue samples. In this respect, Ginsberg *et al.* [68], discovered that compared to normal CA1 neurons, those neurons from the brains of Alzheimer's disease patients with neurofibrillary tangles displayed significant decreases in the mRNAs that encode proteins implicated in Alzheimer's disease neuropathology, including glutamate receptors, dopamine receptors, cytoskeletal proteins, synaptic proteins and phosphatases/kinases. Furthermore, Whitney *et al.* [69], used cDNA microarrays to identify many genes not previously associated with the mechanisms underlying multiple sclerosis.

Of relevance to severe psychiatric disorders, Mirnics *et al.* [50], reported the use of cDNA microarrays to discover that transcripts encoding proteins that contribute to presynaptic function are downregulated in the prefrontal cortex of schizophrenic subjects. These results are consistent with alterations in the prefrontal cortex as contributing to the cognitive disturbances that are characteristic of this disease. Mirnics *et al.* [70] later reported that 'the transcript encoding regulator of G protein signaling 4 (RGS4), was the most consistently and significantly decreased in the prefrontal cortex of all schizophrenic subjects examined.' In addition, Hakak *et al.* [71] have also used DNA microarrays (Affymetrix) to analyze genome wide mRNA expression changes in postmortem tissue from the prefrontal cortex of chronic schizophrenia patients. Interestingly, a group of myelination associated genes exhibited decreased expression in this patient cohort suggesting that oligodendrocyte function in schizophrenics could be disrupted. Traditional techniques such as *in situ* hybridization [50,70], or RT PCR [71] were used by a number of these investigators to validate their DNA microarray results. These studies support the potentially important contribution that the use of gene expression profiling will make to the understanding of human brain disorders. Results from these studies could lead to new directions for research on these brain disorders and possibly the identification of novel targets that could facilitate the development of better therapeutics for these diseases.

Animal models of CNS disorders

Animal models of human disease provide a valuable means to address the mechanisms underlying these diseases. Human brain tissue can be difficult to obtain and, as previously described, there are many limitations to the use of human brain tissue in the analysis of changes in mRNA expression. Importantly, animal models allow for the use of controlled manipulations. For example, Niculescu *et al.* [72] recently reported the application of oligonucleotide GeneChip microarrays to an animal model of mania and psychosis. Rats were treated with methamphetamine and gene expression changes measured in two brain regions. A novel aspect of this work was the comparison of the map position of the human homologues of genes whose expression was changed in the animal model to genomic regions implicated in linkage studies of bipolar disorder and schizophrenia. Using this approach, termed convergent functional genomics, a smaller number of higher probability candidates can be identified for further study among the sometimes hundreds of genes within linkage hotspots. They identified a gene, G-protein-coupled receptor kinase 3 (GRK3), which underwent a large increase in expression in the animal model and mapped precisely to a region on human chromosome 22 implicated in linkage studies of both bipolar disorder and schizophrenia. Such an integrative strategy, combines the strengths of these two genomic approaches (positional cloning and functional genomics) in order to identify disease genes. However, a significant limitation is that the

expression of a disease susceptibility gene may not be altered in either the human pathophysiology or in an animal model even though its mutation affects the expression of other genes. Nevertheless, this approach may help identify a subset of disease genes whose transcriptional regulation is altered by mutation.

Of relevance to neurodegeneration, Lee *et al.* [73] reported the use of oligonucleotide arrays to investigate genes associated with aging in mice and discovered that the aged mouse brain exhibits changes that parallel those observed in human neurodegenerative disorders. These results were confirmed by RT-PCR. In addition, rodent models of neurodegenerative diseases such as Parkinson's disease and Huntington's disease [74-76] have been developed. DNA microarrays have been employed to analyze global patterns in gene expression, in at least one of these animal models [77,87], facilitating a better understanding of potential functional changes in these disorders. However, one of the major limitations of the use of animal models is that it is not clear that the underlying pathology in the animal model is the same as in the human condition.

Animal models can also be very useful for investigations into the effects of the drugs used to treat these diseases. Additionally, changes in neural gene expression induced by these drugs could provide insight into the mechanisms underlying the disorders for which these drugs have therapeutic efficacy.

Cell culture models: neural cell lines

The use of cell culture models to identify global gene changes represents a powerful means to analyze gene expression changes affected by various manipulations in a closely controlled environment. Some potential uses of DNA microarrays with cell lines to investigate brain disorders are discussed in this section.

Gene profiling of patient lymphoblastoid cell lines in both resting state and after drug challenge

Primary brain tissue cannot be used for investigations into mRNA changes during the life of the patient. In this case lymphoblastoid cell lines can be used to access patterns of gene expression. The primary problem with this approach is the limited way in which lymphoblastoid patterns of gene expression resemble those in the brain. Yet for some small subset of genes, this could be a useful way to access gene expression in a tissue that is readily available.

The use of neurally derived cell lines

Neurally derived cell lines have the advantage of representing homogeneous cell populations and being readily manipulated. They include naturally or artificially transformed cell types such as neuroblastomas, as well as cells induced to follow specific differentiation paths by neurotrophic factors. Such models have the ability to measure genes relevant to specific neuronal functions in isolated systems. Scarlato *et al.* [78] have reported the use of expression profiling on oligodendrial progenitors. This profile of gene expression changes could be of relevance to neurologic disorders such as multiple sclerosis, which is characterized by degeneration of myelin (oligodendrocytes).

The use of heterogeneous stem cell culture systems to analyze gene expression changes in CNS progenitors

Geschwind *et al.* [79] used representational difference analysis (RDA) subtraction, on a heterogeneous stem cell line, to derive microarrays used to study gene expression pattern alterations in CNS progenitors. This combination of methods allowed for the discovery of both known and novel enriched genes expressed in neural and hematopoietic stem cells suggesting common gene expression changes in these two types of progenitors.

Other non-array approaches

Chemical mutagenesis

Chemical mutagenesis has long been used in *Drosophila* as a forward genetic method to identify genes associated with specific phenotypes. Recently, this approach has been applied to mice on an increasingly large scale. Mice are treated with chemical mutagens such as ethylnitrosourea (ENU) in order to induce mutations. The animals are then screened for phenotypes of interest and bred in order to isolate specific mutations and verify their heritability. Once a mutant for a phenotype of interest has been obtained, the gene is isolated by positional cloning methods. The power of ENU mutagenesis combined with the ability to clone murine genes by map position provides a generally applicable approach to study complex behavior in mammals.

One of the most notable applications of this approach in mammals was in a search for genes that regulate circadian rhythms. The progeny of mice treated with ENU were screened for circadian clock mutations. A semidominant mutation, Clock, that lengthens circadian

period and abolishes persistence of rhythmicity was identified. Clock segregated as a single gene that mapped to the midportion of mouse chromosome 5, a region syntenic to human chromosome 4 [80].

Nolan *et al.* [81] reported the use of chemical mutagenesis in a large scale effort to identify a variety of CNS mutants. They screened over 26,000 mice and identified 500 mutants with different neurological phenotypes of interest. This collection of mutants provides a powerful resource for studies of brain disorders. 'Phenotype driven mutagenesis' will facilitate the discovery of novel gene mutations that are associated with novel phenotypes. Since there are no *a priori* hypotheses in this type of study, novel genes involved in biochemical pathways contributing to behavioral phenotypes can potentially be discovered.

Transgenics, knockouts, knock-ins and antisense

The use of transgenic mice provides an *in vivo* means to link DNA sequence to biological function. For example, novel transgenic strategies have been developed to take advantage of the large number of cDNAs that have now been identified. One of these approaches utilizes *in vivo* libraries of transgenic mice [82,83]. This approach makes use of large inserts that contain megabases of DNA that map to a genomic region associated with the phenotypes of interest. This approach has led to the discovery of genes underlying learning defects [84-86] and neurodegenerative disorders in mice [87-89]. Novel high-throughput strategies such as the application of *in vivo* libraries to transgenic mice will facilitate the linking of human DNA sequence to function [82].

Another new transgenic strategy utilizes full-length cDNAs that correspond to expressed sequence tags (ESTs). Constructs containing these cDNAs are introduced individually into the mouse genome and the resulting transgenic mice are analyzed for various behavioral phenotypes [90]. In this way the function of ESTs can be addressed [82].

There are many limitations for the use of transgenic mice as a functional genomics strategy. One of the major limitations of the use of animal models is that it is not clear that the underlying pathology in the animal model is the same as in the human condition. Additionally, mice have small litter sizes, long reproductive cycles and are expensive to maintain [82].

However, even in light of these limitations, mice remain the organism of choice to model human disorders, particularly neurogenerative brain disorders. Mice have been used to gain an insight into the neural alterations in Alzheimer's disease and amyotrophic lateral sclerosis [91].

Transgenics, knockout, knock-in and antisense technologies can also be used in conjunction with DNA microarrays to investigate global gene expression changes in mice that either overexpress, underexpress or do not express a given gene. From these studies gene expression patterns can then be identified that are altered by shutting off or by turning on specific genes. These techniques are usually limited to the analysis of one gene alteration at a time. In such cases, they still provide powerful complementary functional information to the functional genomics approaches we have previously described.

Applications of DNA microarrays to drug discovery of relevance to brain disorders

Functional genomic approaches have also simplified the drug development process. In this regard, drug discovery programs at biotechnology companies are using custom microarrays to isolate lead compounds [23]. Cells in model systems of interest can be treated with known efficacious drugs for specific diseases and the patterns of gene expression induced by these known compounds can then be compared to those induced by the compounds under investigation. In this way a compound can potentially be screened by parallel gene expression pattern induction.

Furthermore, if gene expression patterns that are associated with the unwanted side effects that result from conventional drug treatment are isolated, lead compounds can be screened to identify potential drugs that do not induce these gene expression patterns. This process would facilitate the development of therapeutically efficacious drugs that lack side effects.

DNA microarrays can also be used for drug target validation as well as the discovery of secondary drug targets [18,23]. An alternative way to address the mechanisms underlying brain disorders is to use microarrays to analyze gene expression data that result from drug treatments that are used as therapeutics for a brain disease. Such an approach could potentially be very powerful in advancing our understanding of the etiology of complex brain disorders such as psychiatric diseases. For example, evaluating antipsychotic drug-induced gene expression remodeling in brain regions that have been implicated in psychosis could have

Highlights

- Functional genomics describes studies directed at the process of determining the function of genes and how the genome works together to generate whole patterns of biological function.
- One of the greatest challenges of functional genomics is to understand how alterations in known and unknown genes contribute to the mechanisms underlying brain disorders.
- To understand the alterations in genetic programs of relevance to brain disorders it is necessary to utilize technologies that can address multiple changes in gene and protein expression in various brain regions, simultaneously.
- DNA microarrays are the most powerful of the techniques that address global functional genomics questions. It allows for the expression analysis of thousands of genes in a single experiment, thus facilitating the study of global gene patterns that are affected in symphony by specific diseases and drug treatments.
- Proteomics, the analysis of global changes in protein expression complements functional genomic approaches aimed at profiling RNA alterations.
- One of the main benefits of DNA microarray technology may be identifying the secondary consequences of the primary underlying disease effects on gene expression.
- Gene expression patterns that are associated with a specific disease could potentially be used as diagnostic markers for the disease.
- An alternative way to address the mechanisms underlying brain disorders is to use microarrays to analyze gene expression data that result from drug treatments that are used as therapeutics for a brain disease.
- Functional genomics will facilitate the identification of thousands of new potential targets for drug discovery.
- DNA microarrays can also be used for drug target validation.

implications for those genes that are involved in the development of schizophrenia and/or manic depression associated psychosis.

Conclusion and expert opinion

The completed draft of the human genome sequence and the recent development of technologies that allow for expression profiling of thousands of genes simultaneously in the brain has revolutionized our ability to address mechanisms underlying human brain disorders. Previously, gene expression studies were limited to the analysis of one or a couple of gene expression changes. Now global changes in gene expression can be investigated and common functional attributes of gene alterations can be identified in human brain diseases. Microarray techniques do not replace existing techniques but must be used in conjunction with more conventional techniques to validate gene expression changes (RT-PCR, Northern blotting and RNase protection) and to gain cell type and regional specificity (*in situ* hybridization) and parallel information on protein expression (western blotting and immunohistochemistry) [11].

One of the main benefits of DNA microarray technology may be identifying the secondary consequences of the primary underlying disease effects on gene expression. Alterations in gene regulation are most likely the secondary consequence of the disease state. The actual cause of a genetically transmitted disorder is mutated genes. Even if these gene expression changes are a consequence of the disease state they could contribute to the long-term expression of a brain disorder as well as the inability for certain patients to remit. Hence, microarray studies may contribute not only to identifying disease genes, but also to understanding the different pathophysiologies resulting from different disease genes.

One of the great challenges of these functional genomics approaches is to make biological sense of the vast array of data that is generated by these techniques. Advanced data mining tools have been created to address these problems and this software has been and will be improved as greater numbers of genes are isolated and the knowledge of the complexity of gene interactions is expanded.

Changes in gene and protein expression, identified by functional genomic and proteomic techniques, in the brains of patients with neural diseases will facilitate the identification of drug targets for many brain diseases. In this way, new and improved treatments for brain diseases, especially severe psychiatric disorders such as schizophrenia and bipolar disorder could be developed that would help those suffering from these diseases.

Outlook

Within the next 5–10 years DNA chips representing the entire genome will be available allowing investigators to analyze alterations in the expression of all human genes expressed in the brain. This technological advance will allow scientists to potentially determine how gene product interactions in different brain regions contribute to human brain diseases. These discoveries could also be used as gene expression 'signatures' to facilitate the diagnosis of specific brain disorders.

Identifying gene expression changes in human brain disease is only the beginning. One of the greatest challenges of functional genomics is to understand how alterations in known and unknown genes contribute to the mechanisms underlying brain disorders. Genes identified as differentially expressed in diseased *versus* control brains, animal models or cell lines will become candidates for more focused biochemical and molecular analysis. Advanced data mining tools that will enable scientists to better link sequence to function will be developed.

To address the etiology of human brain disorders proteomics and other approaches will be needed to complement results from functional genomics studies in order to gain a more complete understanding of the contributions of the protein activity encoded by the genes of interest. Functional genomics, proteomics, metabolomics and regulomics [92] (analysis of promoter function) will be used together to understand the interactions between gene expression, protein mechanisms and metabolism. These methods will also be used in concert to understand the complete metabolism and function of all proteins in the brain facilitating our understanding of human brain disease.

In summary, within the next 5–10 years functional genomics will facilitate the identification of many new disease genes and drug targets. As a result better drugs with less side effects (e.g., severe psychiatric disorders) will be developed and diagnostic tests for complex brain disorders should become available.

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