Profiling brain transcription: neurons learn a lesson from yeast

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The application of microarray technologies to the brain poses unique challenges, because of the complexity of the central nervous system and the availability of resources. Nevertheless, recent studies using DNA chips have made inroads into the molecular characterization of regional and functional brain units, the identification of developmental gene expression patterns, and the discovery of transcriptional differences associated with behavioral and neuropathological traits.

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Abbreviations
CNS central nervous system
EST expressed sequence tag
QTL quantitative trait loci

Introduction

DNA microarrays have become an integral part of the research tools used in the laboratory, and have been avidly adopted by molecular neurobiologists. Boosted by the completion of genome sequencing projects in various model organisms and in humans, DNA chips hold great promise for the systematic measurement of complete transcriptional programs in any tissue or cell type, and at any stage of a given physiological, developmental or pathological process. As we have learned recently from studies on yeast, expression profiling provides access to entire regulatory mechanisms and networks in any given physiological process by revealing concerted and genome-wide changes in transcription [1–4]. In doing so, large-scale expression analysis reveals interrelated processes, for example the upregulation of transcripts involved in wound healing during the physiological response of human fibroblasts to serum [5]. Furthermore, by inferring that the relative abundance of specific transcripts is a response to specific cellular needs, the function of many previously uncharacterized genes, whose expression level tightly correlates with the time course of a specific event and transcriptional changes in known genes, has been tentatively predicted. Finally, the global transcription profile itself is a direct representation of cellular phenotype. By providing a complex, but accurate cellular identification, gene expression profiling leads the way to the generation of new diagnostic and prognostic tools, as recently demonstrated for tumor classification [6••–8••].

These characteristics make biological discovery with microarray analysis extraordinarily tantalizing for molecular neuroscientists. The ability to monitor in parallel the expression of tens of thousands of transcripts in each biological sample is likely to provide insightful information into issues of extreme complexity: the cellular commitment to various neuronal cell fates; the formation and maintenance of appropriate neural connections; the identity of neuronal circuits involved in cognitive processes and behavioral arrays; and the identity and progression of neurological diseases, being some examples.

When, then, will the neurochip revolution occur? Following a relatively late start compared to other fields in biology [9], a small but increasing number of publications report the transcriptional profiling of neurons. As we discuss in this review, data extracted from these initial microarray analyses represent useful proof of principles that enlighten the specific challenges and unique promises of large-scale profiling of brain transcription. For a more general description of microarray technology, its method of analysis, and its fields of application outside neuroscience, the reader is referred to a number of recent reviews [10–12].

Building a molecular map of the brain

Molecular characterization of brain units

The vertebrate brain is subdivided into anatomically and functionally distinct regions and nuclei. Stereotaxic lesions and, more recently, functional imaging have correlated specific cytoarchitectonical units with defined physiological functions. The molecular characterization of these cellular units is now a critical step for further functional analysis. Knowledge of the entire molecular composition of a given brain compartment — its specific set of neurotransmitters and associated processing enzymes, neurotransmitter receptors, ion channels, growth factor receptors and regulatory molecules — will indeed significantly help to define its function and to identify brain-specific therapeutic targets. Moreover, and of prime importance in experimental neuroscience, molecularly-defined brain units will become amenable to genetic manipulations: the targeting of reporter genes and/or inducible targeting cassettes into gene loci with brain region-specific expression patterns will permit the direct visualization of specific neuronal ensembles and their synaptic contacts, and will provide an experimental system to precisely assess gene function in defined brain areas.

Methodological considerations

Two studies [13•,14•] have explored differences in gene expression in specific brain regions using DNA chips. In an attempt to identify genetic differences responsible for the distinct structural and behavioral phenotypes of two inbred mouse strains, Sandberg *et al.* [13•] apply microarray technology to assess the expression of >10,000 genes and expressed sequence tags (ESTs) in six different regions in

the brains of 129SvEv and C57BL/6 mice, using the Affymetrix Murine 11K set of high density oligonucleotide arrays. In a similar approach, Zirlinger *et al.* [14•] analyzed differential gene expression among five selected brain regions of the CD1 mouse strain, using the Affymetrix GeneChip arrays, which represent 34,000 genes and ESTs, and have further extended their analysis to genes with restricted expression in the amygdala. From a methodological standpoint, the two studies emphasize the same points. First, despite an expected imprecision in the dissection and in the physiological state of the sample, a high level of experimental reproducibility — <0.02% of genes vary significantly in duplicate profiling of the same brain region — and low background noise levels can be ensured by stringent methodological and analytical methods. Second, the validation of the microarray results with alternate methods such as RT-PCR or *in situ* hybridization on the same experimental material is essential. Indeed, despite considerable data accuracy and reproducibility, in their experimental system, Zirlinger *et al.* [14•] show, by *in situ* hybridization analysis, that 60% of the identified genes are expressed in a manner fully consistent with the array analysis, 20% do not show any signal, 13% hybridize everywhere and 7% are inconsistent with the microarray results. According to the authors, most of the observed inconsistency reflects probes that hybridized everywhere. This can be attributed, at least in some cases, to sub-optimal probe design rather than the inaccuracy of the GeneChip method.

Regional differences

In their analysis of regional brain differences, both approaches reach similar conclusions: on average only 0.3–0.5% of the sampled genes show a significant expression difference — as measured by a ≥1.8 fold [13•] or a ≥3.5 [14•] expression difference — in a given brain region compared to another. Remarkably, Zirlinger *et al.* [14•] find that 75% of amygdala-enriched genes exhibit boundaries of expression within the amygdala that correspond to cytoarchitectonically defined subnuclei, thus demonstrating the feasibility of generating a molecular brain atlas in which each brain structure is defined by a specific molecular identity. As shown with the cancer classification on the basis of gene-expression monitoring [6••–8••], this identity might result from the presence of unique genes or the unique combination of defined sets of genes. Interestingly, with the exception of the cerebellum, most if not all differentially expressed transcripts were found to be either up- or down-regulated from one brain region to the other, rather than being either strictly present or absent in distinct brain regions. This is surprising because the cortex in particular comprises enormous neuronal diversity, thus one might have expected a large set of cortex-specific transcripts. The overall expression level or the number of cells in a given region expressing the differential genes, however, appears rather high, suggesting that the present approach systematically excludes genes expressed at low levels and in small subsets of cells. This might result from either one or both of the following factors: first, the extreme cellular heterogeneity in most brain regions may result in the extreme dilution of cell-specific but rare transcripts, and thus generate an overall lack of sensitivity of the array detection; second, many unknown region-specific genes may exist that are not represented in the available databases and therefore absent from the Affymetrix GeneChip arrays. These limitations may explain the rather counterintuitive results published by Sandberg *et al.* [13•], who show that the cerebellum appears the most molecularly distinct region. Indeed, because the cerebellum contains a relatively small number of neuronal cell types, RNA isolated from it is more likely to contain transcripts specific to each neuronal category. Consequently, cerebellum-specific transcripts are probably abundantly represented in the database as identified genes or ESTs, are therefore more likely to be present on microarrays, and can provide hybridization signals of sufficient intensity for the corresponding gene to be detectable above background. This issue of sensitivity and cellular resolution of microarray experiments will be further discussed in a later part of this review.

Following neurons in their developmental time course, thousands of genes at a time

Developmental time points

Brain development is regulated by intricate signaling cascades and involves ubiquitous neural maturation events, as well as differentiation processes specific to a given neuronal cell type. Direct comparison of the transcriptional profiles of a particular neural structure, at different developmental time points, may permit the characterization of sets of genes that specify its unique identity, and the direct visualization of the coordinated developmental events that are essential for its proper function in the adult brain.

Mody *et al.* [15•] have analyzed the developmental transcriptional programs of the mouse hippocampus from embryonic day 16 to postnatal day 30, using the mouse 11K sets of Affymetrix oligonucleotide array. Experiments were performed in duplicate and led to the identification of 1926 genes or ESTs with three-fold changes in expression level, between at least two to five developmental time points. Data analysis led to the identification of 16 defined groups of genes, with dynamic patterns of expression tightly correlated with major developmental hallmarks, such as neuronal proliferation, differentiation, synapse formation, and synapse maturation and function.

Developmental mutants

A very different type of developmental paradigm has been followed by Livesey *et al.* [16••], who employ a home-made microarray of 960 adult mouse retina cDNA clones to compare the expression profiles of retinas dissected from wild-type and Crx mutant mice. Crx is a photoreceptor-specific homeobox-containing transcription factor that controls terminal differentiation of vertebrate photoreceptors. Crx mutations cause congenital blindness via photoreceptor degeneration. Using this approach, which closely parallels the successful experimental approaches of mutant cells in

yeast [17••], Livesey *et al.* [16••] successfully identify a core set of photoreceptor genes as candidate Crx targets *in vivo*, as well as a novel motif in the promoter regions of prospective Crx target sites.

CNS progenitors

A different but similarly reductionist approach identifies genes with enriched expression in cultures of central nervous system (CNS) progenitors [18••]. In order to focus and enrich their search for specific gene expression by CNS stem cell subpopulations, the authors use a multistep approach. First, a subtraction was performed between cultures of neurospheres, thought to contain 3–4% totipotent stem cell progenitors, and cultures left to differentiate for 24 hours, which are thus likely to be depleted of progenitors. In a subsequent step, 6000 cDNA species, including those isolated from the subtraction product, were spotted onto glass slides and hybridized with neurosphere and differentiated culture cDNAs. From this experiment, 79 unique sequences were found to be enriched >3.3 fold in the precursor pool and were further analyzed. Partial confirmation of the array data was performed by Northern blot and *in situ* hybridization, demonstrating a selective enrichment of the identified genes in the germinal zone of the mouse brain.

Differences in experimental approach

It is interesting to compare the outcomes of the three different experimental approaches. Mody *et al.* [15•], by undertaking a wide screen for developmental genes, have identified a very large panel of potentially interesting transcripts, but the current lack of analytic and/or experimental tools to directly and quickly address the importance of these genes at specific time points of development restrict them to a preliminary description of the microarray data. Noticeably, the authors face an exceedingly large set of data that includes a large proportion of genes with uncharacterized function. Consequently, and while awaiting more substantial but long-term analysis, the authors have organized candidate genes according to known developmental categories, and thus are not able to easily extract substantially novel information from their primary data. In contrast, the last two studies lead a focused search for differentially expressed transcripts, by virtue of a tight comparison between mutant and wild-type tissue [16••] or by the selective enrichment of desired transcripts [18••]. Here, the experimental design restricts the search to genes with a desired function. These studies provide interesting examples of combining modern microarray technology with traditional hypothesis-driven approaches in neuroscience.

A step towards the study of complex phenotypic traits and neurological diseases

The ability to follow orchestrated and genome-wide gene expression has led several groups to identify a complex issue at the core of brain function: can one identify transcriptional differences associated with specific behavioral or neuropathological traits?

Gene expression and seizure susceptibility

An initial contribution to this issue comes from the comparison performed by Sandberg *et al.* [13•] of expression profiles of distinct brain regions in two inbred mouse strains, known to display significant differences in seizure susceptibility and behaviors. Through their microarray analysis, the authors identify 24 genes that are differentially expressed between the two mouse lines in all brain regions examined. In addition, ~50 other genes appear differentially expressed between the two mouse lines in some, but not all, brain regions. In fact, the distinction between these two categories of genes seems quite superficial, as the authors note a general trend for these genes to have a different general expression between the two strains in all brain regions. A handful of genes are also shown to have different degrees of up- or down-regulation during seizures, but no gene was found to be oppositely regulated in the two strains. Clearly, and for the reasons stated earlier, many potential gene candidates may have been missed due to the relative lack of sensitivity inherent to such an approach. Are the genes identified to date relevant to the known behavioral and disease susceptibility strain differences? As noted by the authors, data obtained by microarray analysis are only correlative, and as a first step, only provide a substrate for educated guesses.

More interesting, perhaps, is the observation that several differentially expressed transcripts are encoded by chromosomal regions thought to harbor genes important for strain differences in CNS phenotypes. Thus, as emphasized by Sandberg *et al.* [13•], the combination of expression profiling and quantitative trait loci (QTL) analysis in mice is likely to provide a tremendous synergy for the identification of candidate genes responsible for quantitative traits and disease. Indeed, expression profiling may provide an efficient method to identify a set of genes involved in specific complex traits, and thus nicely complements QTL analysis, which is able to map susceptibility loci to defined but large chromosomal intervals.

Additionally, several studies have followed gene expression in brain regions of aging mice, mice raised in an enriched environment and mice treated with the prospective anti-aging dietary supplement gingko biloba [19–22]. These studies note the correlated fluctuations of key genes involved in neuronal structure, synaptic transmission and plasticity, apoptosis and neuroprotection, all of which are likely to contribute to variations in cognitive processes. For the most part, similar comments can be made about these data as have been made for non-hypothesis driven, genome-wide developmental searches: the gathering of large data sets, including many genes of unknown function, together with the lack of immediate experimental validation tools, leads to a crude description of genes cataloged by known function. Direct experimentation or additional validation tools will be required to provide novel insight into the brain function of interest.

Human neuropathological processes

Some human neuropathological processes have begun to be analyzed by microarray analysis [23,24•,25,26,27•,28,29]. The etiology of many neural diseases remains elusive and often appears multifaceted, with genetic, developmental, nutritional, and environmental factors. Advances in gene analysis have allowed scientists to systematically study the genes altered in pathogenesis and provide testable hypotheses for further study. For example, two groups [24•,27•] have recently identified genes associated with schizophrenia, a severe and complex psychiatric disease. Both groups use microarray technology to study the global gene profiles of prefrontal cortex from diseased and control populations. In one study, Hakak *et al.* [24•] compare 12 sample pairs for the expression profile of >7000 human genes using the Affymetrix HuGeneFL chips. They find that some myelination-related genes are dramatically down-regulated among the patients, thus suggesting that oligodendrocytes are functionally deficient in schizophrenia. In another study, Mirnics *et al.* [27•] investigate six pairs of samples using the Incyte UniGEM-V high-density cDNA microarrays, containing >7000 human genes. In contrast, they find a reduced expression of genes related to the presynaptic secretory machinery in schizophrenia patients. It is interesting that there seems to be no overlap between the findings of the two groups. It is possible that the two types of commercial arrays chosen by the two groups have different gene representations and that the distinct analytic tools led to distinct interpretations. More likely, given the polygenic nature of many neurological diseases and the very different type and age of the patients analyzed in the two studies, the samples analyzed by the two groups may represent different subtypes of schizophrenia. These studies raise the need to have the entire human genome on a single set of arrays, and illustrate the importance of sample selection and grouping processes for appropriate data gathering and interpretation in complex disease studies.

Are existing microarrays adapted to the needs of neuroscience?

An immediate problem in neurogenomic studies arises from the fact that public databases are biased toward most abundant genes and ESTs but, because of the complexity of the nervous system, neuronal-specific genes are either less abundant or only expressed in specific cell types. For mammalian species, in contrast to organisms like yeast and *Caenorhabditis elegans*, most commercial microarrays are designed on the basis of known genes and ESTs, and not on whole-genome information, and therefore many neuronal-specific genes are likely to be significantly under-represented on so-called genome-wide expression arrays. Some neurobiology arrays have been generated to compensate for the general non-neuronal bias of commercial arrays. Affymetrix's GeneChip rat neurobiology U34 array contains >1200 genes involved in the function of the nervous system. Similarly, Clontech has developed the Atlas human neurobiology array, which includes 588 genes related to brain function. In addition, many laboratories have generated their own arrays based on cDNA libraries of nerve tissues or cells [16••,30]. For example, scientists at Millennium Pharmaceuticals have fabricated a brain-biased cDNA array of >7500 cDNAs derived from libraries of rat frontal cortex and nerve growth factor-deprived differentiated PC12 cells [30]. By using this brain-biased array, the authors have identified genes that are regulated during programmed cell death in cerebellar granule neurons. Finally, in order to orchestrate the discovery of more neuronal-specific genes and expression patterns, the National Institute of Mental Health and the National Institute of Neurological Disorders and Stroke initiated, in 1998, the Brain Molecular Anatomy Project (http://trans.nih.gov/resources/resources.htm). This initiative should allow better representation of neuronal genes and ESTs on commercial and 'home-made' microarrays, together with a more accurate analysis of gene expression pattern and function in the brain. Ultimately, together with the knowledge of the whole mouse and human genome sequences, the systematic identification of all neuronal transcripts, including the expected large variety of splice variants in the brain, will permit a more comprehensive and subtle approach to the analysis of brain transcriptional profiling.

Switching from multi- to single-unit molecular recording

One of the biggest challenges for microarray technologies in the neurosciences is how to deal with the marked cellular heterogeneity and low expression levels in many genes of the nervous system. Existing techniques require large amounts of starting materials. In general, a few million cells are needed to obtain enough RNA for a single array experiment. However, nervous systems are often composed of heterogeneous cell populations that are difficult to distinguish on the basis of their location and morphology. Decades of single cell resolution in electrophysiology studies have proven that even adjacent neurons with identical morphologies may have remarkably different physiological responses to the same stimulation. In addition, in studying neurobiological diseases, pathological cells are often surrounded by normal cells. It is therefore necessary to dissect the nervous system into units of lesser complexity in order to obtain meaningful molecular fingerprints of certain cell types or even single cells.

How does one obtain relatively pure samples from complex systems, and how does one faithfully amplify RNA transcripts in order to meet the minimal requirement of array study? Subtraction is a popular technique to enrich transcripts specific for an interesting group of cells. For example, in order to study the gene expression of CNS stem cells with microarrays, Geschwind *et al.* [18••] first applied representational difference analysis subtraction to enrich the CNS progenitors in a heterogeneous stem cell culture system. Libraries made from subtractions were then used as targets for array studies. In addition, recent advances in isolation or in enrichment for the cell types of interest, such as fluorescence activated cell sorting and laser capture microdissection, may allow the researcher to reduce the complexity of samples significantly and enrich

for a preferred neuronal cell type. In this respect, the expression of fluorescent reporter genes in targeted or restricted neuronal populations will be of great use (see in particular, the use of novel transgenic tools with direct applications in neuroscience as described in [31,32]).

Great hope lies in the possibility of faithfully amplifying cellular RNAs from a few or even single neurons, thus reaching the ultimate resolution and sensitivity of the smallest brain functional unit. In the past decade, extensive efforts have been made to identify and develop faithful amplification methods. To date, two major techniques are available. The first is an *in vitro* transcription-based linear amplification or antisense RNA amplification method, pioneered by Eberwine [33], to successfully detect gene expression in single neurons ([33,34]; see also [35,36] and recent improvements in sensitivity and resolution [37,38]). The second, alternative approach, is a polymerase chain reaction-based amplification method [39,40], which has proven extremely efficient in traditional cloning and expression analysis at the single-cell level. This technique, at present under investigation in our labs using high-density oligonucleotide arrays, should increase the sensitivity of detection to a few copies of transcript per cell in single cells. If faithful representation and transcriptional complexity of the original cell transcripts are kept, these approaches should hold great promise for the detection of unique transcripts involved in defining a particular neuronal cell type in a specific brain nucleus.

Conclusions: complex system, complex tools, complex message

A survey of the literature on mammalian brain transcriptional profiling together with comparisons with advances in other experimental fields and organisms underlines some specific challenges for the use for microarray technologies in neuroscience. Not surprisingly, when studying a complex experimental system such as the brain, with a genome-wide analytical tool, the message provided appears extraordinarily complex. The question now is whether we have the right means to decode this message, and if not, what are the options to improve the resolution of neuronal profiling?

Microarray experiments, in particular in yeast and in other simple systems, rely on non-hypothesis driven approaches. These consist of genome-wide gene expression analyses without any preconceived hypotheses, uncovering novel and significant biological processes and leading, in turn, to the formulation of new hypotheses and experiments. However, this might not apply very well, at least not yet, to the field of neuroscience, because the data obtained from neuronal profiling are too partial and too complex to be directly insightful. On one hand, the detection of relevant transcripts is significantly impaired in most experiments because brain tissue is extremely heterogeneous, a factor that drastically decreases both the sensitivity and the resolution of the analysis, and because the microarrays themselves are in most cases not optimized for neuroscience studies. On the other hand, profiling analysis generates an enormous amount of raw data, and includes the transcriptional behavior of large sets of uncharacterized genes. The meaningful use of these data will require the availability of systematic analytical tools: powerful computational and statistical algorithms, cross-analysis of data obtained in different experimental systems and different labs (see, in particular, the interesting discussion on data sharing in [41–44]), larger collections of behavioral and neural mouse mutants, more complete gene expression patterns and gene function databases, as well as various *in vitro* assays of neural function. Although efforts in all these directions are underway, one should remember that, in addition to the small size of its genome, the success of expression profiling in yeast has resulted from the availability of synchronized and homogeneous populations, and from the generation of large sets of mutants [17••]. Similarly, a survey of the neurochip literature shows that one of the most encouraging and immediate ways to make neuro-array experiments more fruitful, is to reduce the complexity of the starting material to a homogeneous, enriched population, even, if possible, to a single cell, and/or to focus the search by a reductionist approach, for example by comparing samples obtained from a wild type and a mutant with a known neural defect. Clearly, the yeast teaches a lesson to the neuron: get simple and get genetics!

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