Single-Cell Molecular Biology: Implications for Diagnosis and Treatment of Neurologic Disease

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The continued discovery of basic pathologic mechanisms underlying neuropsychiatric illnesses will be critical to the development of improved diagnostic tests and more targeted therapeutic strategies. Molecular biological methods capable of evaluating gene expression at the single-cell level have great potential for advancing our knowledge of these processes. This review describes two techniques that are providing new insights into the intracellular regulation of ribonucleic acid trafficking and processing. These technologies promise to accelerate our understanding of both normal and abnormal molecular processes within neurons, and they have the potential for direct application to the study of human neurologic disease. Biol Psychiatry 2003;54:413–417 © *2003 Society of Biological Psychiatry*

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Introduction

The diagnosis and treatment of neuropsychiatric illness can be a significant challenge, because the etiologies of most neurologic diseases remain unknown. In practice, critical diagnostic tests are not always available or feasible, markers of early disease presence or progression are rare, and the inherent complexity of neurologic diseases, as well as their propensity to "overlap" both pathologically and clinically, often makes definitive diagnosis difficult when it is based solely on clinical grounds. Despite these difficulties, decisions regarding treatment must be made based on experience and available data, and therapy is often initiated to cover a variety of diagnostic possibilities rather than being precisely tailored to a specific pathologic process. Furthermore, few modern therapeutic regimens against neurologic illness are truly disease modifying, or "curative," and many exhibit only marginal efficacy with respect to symptom control. Although we have clearly made significant advances in the management of these diseases thus far, improved diagnostic and therapeutic regimens will only come from better clarification of critical underlying mechanisms of disease.

Pathologic processes in the nervous system cover a broad spectrum: some appear to principally involve abnormalities of neurophysiologic function (e.g., essential tremor, dystonia, depression, and schizophrenia), some are almost entirely the result of cell death (e.g., cerebral infarction), and others involve a combination of these processes (e.g., neurodegenerative disease). Further complexity is added by the fact that pathology is often restricted to subsets of cells in an anatomically defined, disease-specific manner. For example, Parkinson's disease is characterized mainly by dysfunction and degeneration of dopaminergic neurons in the ventrolateral tier of the substantia nigra pars compacta (SNpc); however, it is clear that dopaminergic neurons in other subregions of the SNpc, as well as specific neurons in other brain regions, are also affected (although to a lesser degree), whereas the majority of neurons in the brain appear to be unaffected. In addition to cell type– and anatomic region–restricted abnormalities, many diseases show evidence of subcellular pathology within affected cells. For instance, dendritic pathology is clearly evident in degenerative conditions, such as Parkinson's disease and Alzheimer's disease, and synapse loss is thought to be the most significant histologic correlate of cognitive dysfunction in the latter (DeKosky and Scheff 1990; Irizarry et al 1998). Therefore, future investigation into the etiologies of neurologic diseases should, ideally, be able to compare the cellular physiology of individual affected and unaffected cells, both within and between anatomic regions, in a highly sensitive and quantitative manner.

The most direct way to study gene expression is to assess messenger ribonucleic acid (mRNA). Levels of mRNA can be affected by regulation of transcription, posttranscriptional processing, and degradation. Ribonucleic acid expression can be further modulated by regulation of trafficking between subcellular regions, binding protein interactions, ribosome attachment, and translation into proteins (Crino and Eberwine 1996; Gardiol et al 1999; Garner et al 1988; Kleiman et al 1990; Miyashiro et al 1994). Because it is clear that processes such as these are critical to the maintenance of normal cellular function, it is conceivable that abnormalities in any of these steps

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may be involved in neurologic disease. Below we describe two emerging technologies that should enhance our ability to study RNA-specific molecular processes at the single-cell level: antisense RNA (aRNA) amplification and single-dendrite mRNA transfection. We envision that their application to model systems and human tissues will stimulate new levels of understanding as to the basic mechanisms of neurologic illness.

Antisense RNA Amplification

Because the mRNA complement of a single cell is too small to detect with standard molecular procedures, the RNA must be amplified to measurable levels for subsequent analysis. The aRNA amplification technique is capable of quantitating the abundance of mRNAs in very minute samples through linear amplification of poly-A RNA. As a result, gene expression analysis can be performed at both the single-cell and subcellular levels, allowing comparisons not only between different cells but also between subregions within the same cell.

Antisense RNA amplification capitalizes on the fact that a 3′ poly-A tail is present on most mRNA sequences and that the transcriptional activity of RNA polymerase is linear (Kacharmina et al 1999). Briefly, a first strand complementary deoxyribonucleic acid (cDNA) is reverse-transcribed from mRNA primed by an oligo-dT(24)-T7 polymerase promoter hybridized to the poly-A tail. This procedure can be performed either in situ (on fixed cells) or after sample isolation (on live cells). Single-cell (or cellular process) isolation is effected with the use of a precision pulled glass micropipette filled with either diethylpyrocarbonate (DEPC)-treated water or reverse-transcription buffer. The cell of interest is located on a microscope stage fitted with a micromanipulator, and the target material is dissected and aspirated with the micropipette (Figure 1). Next, the cDNA:RNA hybrid is heat-denatured, and DNA polymerase is used to synthesize the second cDNA strand with a 3′ self-priming loop. The loop is then excised, the double-stranded cDNA is blunt-ended, and unincorporated nucleotides and salts are removed by dialysis against DEPC-treated water. Antisense RNA is then transcribed from the cDNA template with T7 RNA polymerase. The recurrent nature of the transcription reaction at this step results in an amplification of aRNA, in which the relative abundances of mRNAs in the original sample are maintained. If sample sizes are exceedingly small, as they may be in single cells or in isolated cellular processes, pooling of material and repeat amplifications can be performed to increase the probability of signal detection.

The final amplification product is visually assessed with denaturing gel electrophoresis (Figure 2). Intact aRNA appears as a smear that approximates the size distribution of mRNA in the original sample, although the mass average will be slightly smaller, given the 3′ bias of the procedure. Once amplification success is confirmed, the aRNA generated can be used for a variety of applications, including library construction, polymerase chain reaction, and gene expression profiling. For the latter purpose, aRNA can be labeled by various means to probe slot blots, microarrays, or macroarrays (Figure 2).

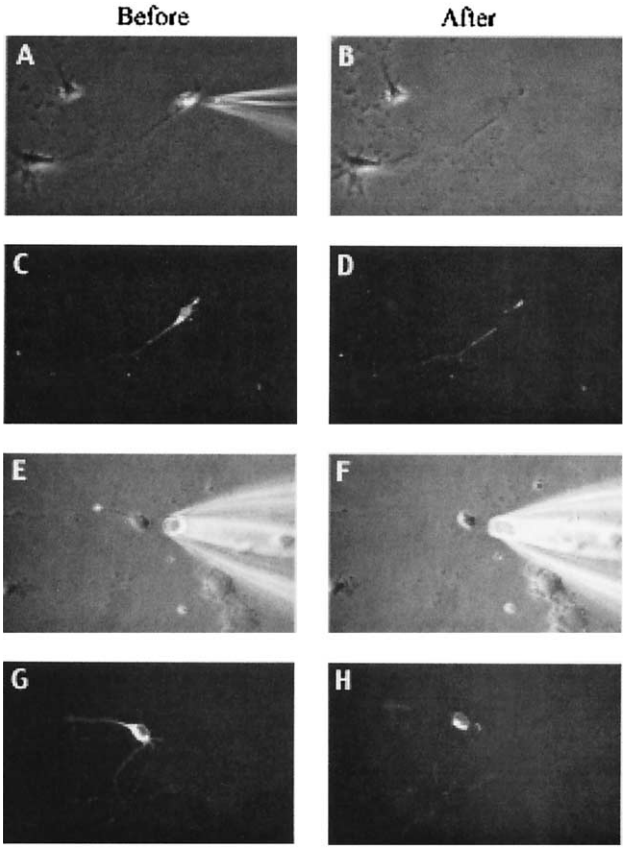

Figure 1. Technique for aspiration of single cell bodies or neurites. Embryonic day 14 mouse midbrain was cultured and immunostained for tyrosine hydroxylase to label dopaminergic neurons. The neurons were visualized by phase-contrast microscopy **(A, B, E, F)** or by fluorescence immunocytochemistry **(C, D, G, H)**. A single cell body (neuron shown in **A–D**) or proximal neurites (neuron shown in **E–H**) were aspirated into a glass micropipette (visible in all phase images except **B**). The left column shows the cell body or neurites just before aspiration, and the right column shows the same cell after aspiration.

This laboratory has used the aRNA amplification technique to study neuronal gene expression profiling at the single-cell level, with a special emphasis on mRNA trafficking to the dendrite. Past experiments have shown that not only are select populations of mRNAs targeted to the dendrite (whereas others are not) but that their relative

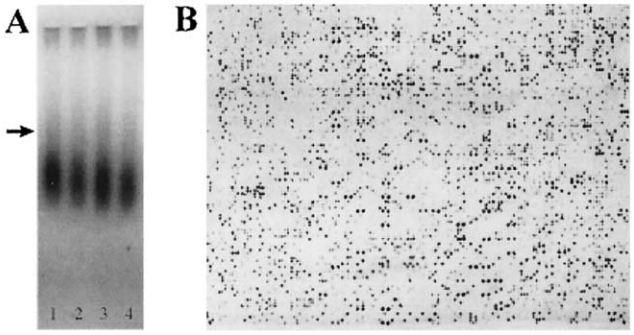

Figure 2. Analysis of antisense ribonucleic acid (aRNA) amplification. **(A)** Representative agarose denaturing gel electrophoresis. Lanes 1 and 3 represent radiolabeled aRNA from eight pooled dopaminergic neurites (each lane) after two rounds of amplification. Lanes 2 and 4 represent the same from five pooled dopaminergic cell bodies (each lane). The arrow to the left represents the migration of the xylene cyanol marker (~4 kb). **(B)** Representative macroarray. Ten pooled hippocampal neuron cell bodies were harvested, and the mRNAs present underwent two rounds of amplification as described in the text. The second round of amplification incorporated P^{33}-labeled uridine triphospate (UTP) and cytosine triphosphate (CTP) into the final products, which were hybridized for 36 hours with a complementary deoxyribonucleic acid macroarray and exposed to a PhoshorImager screen.

abundances can differ within and between processes in the same cell and that their trafficking can be induced and regulated by various types of exogenous stimulation (Crino and Eberwine 1996; Kacharmina et al 2000; Miyashiro et al 1994). Given the fact that polyribosome complexes and other protein translational machinery are present in the dendrite, findings such as these suggest a further level of complexity to the neuronal regulation of gene expression (Crino and Eberwine 1996; Job and Eberwine 2001a, 2001b; Tiedge and Brosius 1996; Torre and Steward 1996).

Single-Dendrite mRNA Transfection

This technique allows specific mRNAs to be transfected into isolated cellular processes to assess mechanisms of in-situ protein synthesis and posttranslational modification. Briefly, capped mRNA is synthesized in vitro and complexed with cationic lipids. The mixture is gently applied with a micropipette onto transected, isolated dendrites, and protein synthesis is stimulated with growth factors. The cultures are then fixed and processed for immunocytochemistry. The development of immunoreactivity for the specified protein product proves that in situ translation has occurred.

Past studies in this laboratory have shown that translation of exogenous transfected mRNA can be stimulated in isolated neurites of single cultured neurons (Crino and Eberwine 1996). Furthermore, these proteins can be post-translationally modified by phosphorylation, and receptor proteins can be both synthesized and inserted into the cell membrane (Crino et al 1998; Kacharmina et al 2000). These techniques could conceivably be modified to further probe the local regulatory mechanisms involved in protein synthesis, processing, and possibly, degradation.

Discussion

Clearly, the potential for techniques such as these is vast, because their ability to assess single-cell physiology is much more sensitive and quantitative than that of most traditional methods. For example, Northern blot and ribonuclease protection methods are quantitative, but they suffer from requiring relatively large amounts of pooled starting material for analysis. Thus, the effects of treatment may be diluted by the presence of RNA from unaffected cells and by the fact that multiple cell types are represented in the final samples (i.e., neurons, glia, blood cells, etc.). In-situ hybridization methods have single-cell resolution and high sensitivity but are not typically as quantifiable as the above-reviewed techniques. These methods also suffer from their inability to use more than a few probes simultaneously, which greatly limits the number of genes that can be studied. Total cRNA prepared from breast tumor biopsy material has recently been used to generate gene expression profiles on arrays that may predict disease outcome at diagnosis (van de Vijver et al 2002). Although this information may aid in the choice of therapy, insights into the underlying neoplastic mechanisms will require identification of the specific cells involved in the expression of these genes. Antisense RNA amplification combines high sensitivity, quantifiability, single-cell resolution, and the ability to screen hundreds to thousands of genes simultaneously, giving it better potential to evaluate the complex, multigenic nature of neurologic diseases. This type of technique is, therefore, more likely to reveal the most appropriate pathways to target for improved symptomatic and disease-altering therapies.

The aRNA technique allows high-fidelity amplification and quantitation of very small amounts of starting material (as low as 1–10 pg of RNA for a single cell). The ability to attain this level of sensitivity and resolution will be critical to the improved evaluation of human neuropathologic tissues, particularly because more precise comparisons between individual affected and unaffected cells, both within and between anatomic regions, can be made. Thus, this technique should help to clarify why only very specific cells and brain regions are involved in certain disease processes. In Alzheimer's disease, for example, the gene expression profile of degenerating cholinergic

neurons in the nucleus basalis of Meynert could be compared with that of neighboring, nondegenerating cholinergic cells within the same brain region, or with affected and unaffected neurons in other cholinergic nuclei (Ginsberg et al 2000). This approach may also be used for more targeted studies of model systems of disease, both in vitro and in vivo. Single living neurons and individual neurites could be identified and studied in primary or organotypic cultures, based on criteria such as cell type, synaptic interactions, association with glia, morphology, growth cone extension or retraction, or gene expression (with markers such as green fluorescent protein). Likewise, fixed or immunostained neurons (and cellular processes) could be identified and studied in cultures or tissue sections by similar features as well as by migration pattern, anatomic localization, antigen expression, degeneration, inclusion body formation, layer of cortical lamination, or fiber input. Any of these models could be applied to the analysis of survival or degeneration in response to various combinations of trauma, toxin exposure, or protective treatment. Furthermore, the ability to separate dendritic from cell soma mechanisms may provide unforeseen insights into the etiology of many neurologic diseases, especially because normal synaptic function is critical to the maintenance of nervous system physiology, and because dendrite-specific degeneration is characteristic of many diseases.

The future may also see this technology automated for diagnosis and therapeutic monitoring in the clinical laboratory setting. The diagnosis of conditions in which pathologic cells may only be accessible in extremely limited numbers, such as carcinomatous meningitis or central nervous system inflammatory diseases, would be greatly augmented by the technique. Pathologic cells from biopsy or body fluid specimens (such as blood, urine, or cerebrospinal fluid) could be assayed for known disease markers to aid in diagnosis of both genetic and acquired diseases. Furthermore, therapeutic efficacy could be monitored by the detection of up-regulation of "protective" genes and the down-regulation of disease pathway genes.

The RNA transfection technique could be applied to the study of diseases in which dendrites are known to undergo pathologic changes early in their course, such as appears to be true in some neurodegenerative conditions. Synapse dysfunction or loss in diseases such as these may be due to altered translational capacity within dendrites or to abnormalities in protein degradation (such as the ubiquitin–proteasome pathway). This, in turn, could lead to inadequate supplies of key synaptic proteins or to their amyloidogenic buildup, respectively. Direct transfection of mRNAs into dendrites in which such processes are suspected, such as in cells from live biopsy material, could provide evidence of abnormal in-situ processing of protein within dendrites and potentially lead to improved diagnostic and therapeutic approaches. Similarly, transfection of individual cell bodies could provide information about somatic mRNA regulation and could be combined with the aRNA amplification methods to detect resultant changes in the expression, trafficking, and processing of other mRNAs.

Some limitations to the techniques described must also be addressed. Given the highly sensitive nature of the methods, the multiple enzymatic and purification steps required throughout the procedure, and the still-emerging nature of the field of bioinformatics with respect to array analysis, a certain level of variability is inherent to the technique. Furthermore, the simultaneous analysis of thousands of genes remains moderately problematic, not only from a statistical standpoint, but also from issues such as background management, inter-experiment variability, and internal standardization of loading. Thus, secondary screens with more traditional methods, such as real-time polymerase chain reaction or in-situ hybridization, must still be used to confirm changes found in specific genes; however, as with the analysis of any biological system, the appropriate repetition of experiments is usually sufficient to separate true effects from those that are more likely to occur by chance. Biological sources of variability are also seen at the single-cell level because of differential expression of mRNAs for specific genes over the length of cellular processes, between different processes, and between different cells. This may pose a particularly significant problem when evaluating tissue sections in which only small portions of cells, or their processes, are available for sampling; however, variability can again be decreased either by judicious repetition of experiments or by pooling of samples for analysis. Signal detection could also be a problem when very small quantities of starting material are used; however, pooling of single cells (or processes) or using multiple amplification steps is usually sufficient for overcoming this obstacle.

In summary, single-cell molecular biology technology is continuing to be developed, and its availability to the general scientific community is increasing. Given the inherent level of sensitivity, quantifiability, and applicability of these procedures, they may be instrumental in revolutionizing our approach not only to the identification of fundamental disease mechanisms, but also to the diagnosis and treatment of neurologic disorders.

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