SECTION COVER FOR THE DEPARTMENT OF NEUROBIOLOGY: Schematic representation of the transcription, transport, and translation of mRNAs that localize to the dendrites of neurons. Neurons, the impulse-conducting cells of the brain, contain a cell body or soma, an axonal fiber that carries the neuron’s output, and one or more dendritic processes, which receive incoming signals at points of neuron-neuron contact called synapses. In neurons, as in all cells, a variety of genes are transcribed into mRNA and translated into protein. Most of this translation occurs within the soma; however, a subset of neuronal mRNAs contains specific sequences that enable the mRNAs to be transported out of the cell body and into dendrites. Translation of dendritically localized mRNAs has been implicated in learning and memory; this local translation is thought to enable synapses to be modified according to their activity, thereby affecting how efficiently they transmit nerve impulses. Members of our department have shown that at least 5 dendritically localized mRNAs have special sequences called internal ribosome entry sites that allow these mRNAs to be translated independently of the cap, suggesting that cap-independent mechanisms may enable the efficient translation of these mRNAs in dendrites. The artwork was done by Vincent P. Mauro, Ph.D.
Chairman’s Overview

For the past decade, the Department of Neurobiology has focused its efforts on primary cellular processes of development, with emphasis on the development of the vertebrate nervous system. This work began with our research leading to the identification and characterization of cell adhesion molecules and studies on factors that regulate cell division. The results of these efforts and the development of new technologies prompted us to examine the control of fundamental processes of gene expression in eukaryotic cells. These processes involve factors that regulate gene transcription as well as those that regulate the translation of messenger RNA into protein. Analysis of these processes has enabled us to examine the development of the nervous system at more fundamental levels. We have, for example, been studying the nature and differentiation of neural stem cells and the molecular mechanisms that underlie long-term potentiation, a key process in memory and learning. Understanding such multilevel integration in complex systems is becoming a crucial challenge in modern biology. Clearly, molecular events are so interconnected that a reductionist approach alone, while valuable, is insufficient for understanding biological systems.

Development and morphogenesis require repeated rounds of differential gene expression. A variety of factors regulates this expression, including elements within the genes themselves and protein factors that bind to the elements. In examining such elements in genes for cell adhesion molecules, Fred Jones and his colleagues discovered a homeobox protein, Barx2, that affects a wide variety of differentiation processes. Exploration of the activity of Barx2 and its targets by Robyn Meech has opened new front lines related to breast cancer, muscle development, and chondrogenesis.

In examining the messenger RNAs expressed in response to neural cell adhesion, Vince Mauro noted that many mRNAs had sequences that matched or were complementary to ribosomal RNA. By analyzing a subset of these sequences, he and his colleagues defined the characteristics of novel sequences within mRNAs that can connect the translation machinery to the message in the absence of the traditional cap sequence. These internal ribosome entry sites (IRESs) were known for viral RNAs but had not been extensively defined in cellular mRNAs. Dr. Mauro and his colleagues went on to show that cellular and viral IRESs differ; the cellular sites are made up of small modules, and the action of cellular IRESs does not necessarily depend on specific secondary structures in the mRNA.

Regulation of translation appears to play an important role in the nervous system, and IRESs may play a special role in the functioning of the synapse. Peter Vanderklish and his colleagues have been studying the factors involved in synaptic events thought to provide the basis of memory and learning in the area of the brain called the hippocampus. Translation of mRNA in dendrites plays a special role in this process. Dr. Vanderklish and his colleagues found that specific messages at the synapse are differentially translated depending on whether the messages are regulated by cap- or IRES-dependent processes. In addition, these researchers are working with Bruce Cunningham and his colleagues to define and characterize granules containing ribosomes, proteins, and mRNAs that are transported from the cell body to the synaptic area in order to allow translation to occur in response to synaptic activity.

The development of the nervous system begins with the differentiation of stem cells to neural progenitors, which in turn develop at the proper time and place into neurons and supporting glia. Moreover, neural progenitor cells remain in the brain in adults, where they presumably repopulate specific brain regions. Kathryn Crossin and her colleagues found that the neural cell adhesion molecule N-CAM can act as a neurotrophin to differentiate the neural stem cells preferentially into neurons.
Moreover, using special multielectrode plates, they defined conditions under which a stem cell population in culture can be converted into neurons capable of exchanging action potentials in a functioning neural network.

All of these activities are aimed at the study of the molecular and cellular events that define and regulate the development of the nervous system. Our efforts have remained focused on fundamental processes rather than on specific diseases. This strategy is based on the belief that understanding even a single primary process can provide the necessary framework for defining the mechanisms underlying not just one but a variety of diseases.

INVESTIGATORS’ REPORTS

Signals and Cell Differentiation Due to Expression and Interaction of Morphoregulatory Molecules


Vertebrate development depends on the interaction and aggregation of groups of cells to form differentiated tissues. These interactions are mediated by classes of cell-surface molecules called cell adhesion molecules. The neural cell adhesion molecule (N-CAM) is expressed on many types of cells during development, most prominently on neurons, astrocytes, and muscle cells, and the aberrant expression of N-CAM is associated with neural disease and degeneration. In addition to delineating its role in cell adhesion, we showed that N-CAM binding leads to alterations in intracellular signaling.

In previous studies, we found that binding of N-CAM to the cell surface of neural stem cells led to enhanced differentiation into neurons that formed functional neural networks with spontaneous electrical activity. More recently, we discovered that the transition to the neuronal phenotype is accompanied by an increase in oxidative metabolism. Newborn neurons produce high levels of reactive oxygen species (ROS) comparable to the levels that occur in some dying cells. Our working hypothesis is that a transient increase in ROS is critical for neuronal differentiation through a variety of intracellular signals.

These signal pathways give clues to the nature of manipulations that may be useful for in vivo cellular therapies. Using fluorescence-activated cell sorting, we sorted cells according to their levels of reactivity with ROS-sensing dyes. The cells with high levels of ROS expressed neuronal markers but not progenitor markers. Among cells with low levels of ROS, 90%–95% expressed the progenitor marker nestin, and more than 95% of the cells incorporated bromodeoxyuridine, indicating that they were proliferating. This system provides a mechanism for isolating highly enriched populations of both neurons and progenitors so that the cells can be recovered and recultured, a goal heretofore difficult to achieve.

In future studies, we will use clonal analysis to determine the developmental potential of each cell population, that is, how many cell types the populations are capable of forming. As in our previous research, to test neuronal function, we will also test the ability of the purified neurons to form networks. In other efforts, we are focusing on the mechanisms by which oxidative state influences neuronal differentiation. The results of these studies promise to link mechanisms of development, aging, and neurodegeneration.

Interrelationships Between Dendritic Protein Synthesis and the Efficacy and Structure of Synaptic Connections

P.W. Vanderklish, F. Smart, G.M. Edelman

The goal of our research is to define the mechanisms by which long-term forms of activity-dependent synaptic plasticity are consolidated. Two basic observations guide our hypotheses. First, translation of dendritically localized mRNAs is required to stabilize changes in efficacy in at least 3 forms of synaptic plasticity: long-term potentiation (LTP), long-term depression (LTD), and synaptic enhancement.
induced by brain-derived neurotrophic factor. Second, each form of plasticity may be associated with unique morphologic changes in dendritic spines.

These observations have led to hypotheses that specific proteins or synaptic shapes are implicated in the expression of stable efficacy states, but it remains unclear how the various changes contribute to stability. Changes in efficacy can outlast the half-lives of new proteins, and distinct proteins may be needed for the functionally divergent plasticities that are coexpressed in neurons having almost 10,000 synaptic connections. We propose that local translation plays a role in transforming synaptic shape and that molecular determinants of synaptic shape in turn regulate the synthesis and localization of proteins (e.g., glutamate receptors) that determine synaptic efficacy. According to this hypothesis, such regulatory interrelationships are unique for each form of plasticity.

Previously, we showed that stimulation of metabotropic glutamate receptors that induce a form of LTD leads to spine elongation and that this effect is blocked by a translation inhibitor. Using real-time imaging techniques, we replicated these data and found in addition that the effect is very rapid and may occur at a subset of spines. These changes resemble the abnormally long and thin spines that occur in fragile X mental retardation, a condition caused by the silencing of a gene, Fmr1, that encodes a translational suppressor found in dendrites. LTD is enhanced in mice that lack Fmr1, and longer, thinner spines contain fewer glutamate receptors. Thus, our data support the notion that translation induced by metabotropic glutamate receptors leads to changes in dendritic spines that express LTD and that this process is not properly limited in fragile X syndrome. This idea and ability of a selective antagonist of metabotropic glutamate receptors to reverse changes in spine morphology are being investigated in cultures of cells from mice that lack Fmr1.

In related research, we obtained evidence that translation in dendrites is reciprocally influenced by determinants of synaptic structure. Using various neuronal preparations, we observed that application of brain-derived neurotrophic factor, which is released during LTP and elicits translation, results in translocation of the eukaryotic initiation factor 4E to synaptic mRNA granules. Depolymerization of F-actin and antagonism of integrins, manipulations that block LTP consolidation, prevented translocation of the initiation factor to granules.

The fact that local translation is involved in stabilizing forms of potentiation and depression implies that such translation is regulated differentially. We are investigating a number of potential mechanisms for differential translation, including heterogeneity of mRNA granules and differential regulation of translation initiation factors by synaptic receptors. Antibodies to mRNA-binding proteins found in granules label distinct particles in dendrites, suggesting that classes of granules exist that are used differentially during changes in plasticity. Related to this finding, we determined that the RNA-binding motif protein 3 is present in a subset of dendritic granules and may be an important regulator of activity-dependent translation. In collaborative studies with B.A. Cunningham and S.A. Aschrafi, Department of Neurobiology, we are further characterizing the structure and composition of granules to address these issues. We also found that synaptic receptors that elicit LTP and LTD have different effects on these initiation factors that influence whether mRNAs are translated via cap-dependent or internal initiation mechanisms. Internal ribosomal entry sites have been identified in some dendritic mRNAs and may provide a basis for selective mRNA use during plasticity. A major goal for the coming year is to further characterize these mechanisms of differential translation and their interrelationships with spine structure.

Protein Interactions in the Nervous System

B.A. Cunningham, A.R. Atkins, S.A. Aschrafi, G.M. Edelman

Critical processes in neural development and neuronal function are regulated by protein-protein interactions and by the binding of proteins to other molecules. We focus on the protein-protein interactions that mediate cell-cell adhesion and on the protein-protein and protein-RNA interactions that regulate the transport of and the time and place at which mRNAs are translated.

Many critical cell-cell interactions involve the binding of a cell-surface glycoprotein on one cell to the same glycoprotein on the surface of another cell. Despite extensive studies, the mechanism by which many of these glycoproteins or cell adhesion molecules mediate this type of cell-cell binding has proved elusive. We have focused on the neural cell adhesion molecule (N-CAM).
To simplify the analysis, we made recombinant proteins corresponding to various combinations of the 5 immunoglobulin (Ig)-like domains and the 2 fibronectin-like repeats that make up the extracellular part of the N-CAM molecule. Each protein has a terminal tag that allows us to attach the molecules to the surface of a polystyrene bead oriented as N-CAM is on the cell surface. Bead-bead binding is then measured in a counter that distinguishes particles of different sizes.

Our results indicate that N-CAM–N-CAM binding requires the distal 2 Ig domains (Ig I and Ig II). The Ig III domain apparently does not act directly in the binding but influences the interaction between Ig I and Ig II. Although smaller proteins can mediate bead-bead binding, optimal binding requires all 5 Ig domains and the first fibronectin repeat. Our current model suggests that the interactions between adjacent domains orient the N-CAM molecule to optimize binding, comparable to what has been suggested for another class of cell adhesion molecules called cadherins. To further test the model, we are comparing the ability of antibodies and other reagents to inhibit the aggregation of beads with the ability of these molecules to block the aggregation of brain cells.

The ability to translate mRNA into proteins at particular times and places is critical for cell function and for development. Recent studies from a variety of laboratories indicated that protein synthesis is required for changes in synapses that form the basis of learning and memory. Moreover, some of this new synthesis takes place locally in dendrites close to the synapses. These and other findings indicate that the mRNAs and the protein synthetic machinery must be transported to and stored in these regions.

Current evidence suggests that mRNAs are transported to the dendrites as RNA-protein complexes of various sizes, including large granules, some of which contain ribosomes. We are working with our colleagues P.W. Vanderklish and F. Smart, Department of Neurobiology, to characterize the composition of such granules and establish the nature of the protein-protein and protein-RNA interactions that organize the granules and the events that activate local protein synthesis. Methods have been developed to obtain fractions enriched in granules. Electron microscopy studies indicate that the fractions contain granules of about 200 nm in diameter; some of the granules contain ribosomes and others do not. These fractions are being monitored for changes induced by drugs or genetic changes that affect synaptic plasticity and by reagents that will be used to further purify the granules.

During these studies, we were using antibodies to the cold-shock protein Rbm3, which is located in granules. We found that some of this protein, however, is tightly associated with ribosomes. Moreover, overexpression of Rbm3 by cellular transfection altered significantly the level of translation as measured by the incorporation of labeled amino acids into protein. Studies are under way to define the mechanism by which Rbm3 affects translation. Because Rbm3 is associated with granules, we are also identifying the mRNAs to which it binds and which part of the molecule is responsible for mRNA binding. We are also characterizing the basic properties of the protein, its ability to form higher order structures, and its ability to interact with other proteins.

Translational Regulation of Gene Expression


We study translational control mechanisms in eukaryotes. Translation begins with the recruitment of the translation machinery by mRNA, which can occur at either the cap, a modified nucleotide at the 5′ end of the mRNA, or at an internal ribosome entry site (IRES) contained within the mRNA. IRESs are a heterogeneous set of sequences, and in earlier studies we showed that some IRESs are composed of shorter functional elements. The mechanisms by which mRNAs recruit the translation machinery and the process by which ribosomes then move to the initiation codon have been the focus of our most recent studies.

In the past year, we completed a study of an IRES module contained within the Gtx mRNA, which encodes a homeobox transcription factor. In addition, our analysis of the BACE1 mRNA, the product of which is involved in Alzheimer’s disease, indicated that the translation of this mRNA occurs by an unusual cap-dependent mechanism in which ribosomes bypass segments of the 5′ leader as they proceed to the initiation codon.

In earlier investigations, we identified an IRES within the 5′ leader of the Gtx mRNA and showed that short nonoverlapping segments of this leader could function as IRESs. We localized the activity of one of these seg-
ments to 9 nucleotides and showed that IRES activity could be greatly enhanced when multiple copies of the IRES module were linked. Most recently, we showed that the 9-nucleotide sequence could bind specifically to 40S ribosomal subunits but not to other cellular components. On the basis of the results of earlier studies suggesting that a segment of the Gtx 5’ leader containing these 9 nucleotides could bind to a complementary segment of 18S rRNA, we tested variations of the 9-nucleotide IRES module for possible correlations between the length of the complementary match, their ability to bind to ribosomes, and their influence on translation efficiency. We found that the length of the complementary match was directly related to the ability of RNA probes to bind to ribosomes and that the ability of this sequence element to enhance translation depended on the length of the complementary match, with maximal translation occurring in constructs containing 7 complementary nucleotides. The results of this study suggest that the Gtx 9-nucleotide sequence enhances translation by a mechanism that involves base pairing to 18S rRNA.

In the past year, we also completed an analysis of the translation of the BACE1 mRNA, which encodes the enzyme β-secretase. In the brains of patients with Alzheimer’s disease, the increased expression of this enzyme is implicated in the overproduction of the β-amyloid peptide, which leads to the formation of amyloid plaques. In this disease, the overproduction of β-secretase occurs without corresponding changes in BACE1 mRNA levels, suggesting that the expression of the enzyme is regulated at the level of translation. These observations led us to examine the mechanism that regulates the translation of the BACE1 mRNA.

Using reporter constructs in transfected mammalian cell lines and cell-free lysates, we showed that the translation mediated by the BACE1 5’ leader is cap dependent and is inhibited by cis-acting segments contained within the 5’ leader. Disruption of 3 upstream open reading frames within the 5’ leader had no effect on translation in B104 neuroblastoma cells. This finding was unexpected because the initiation codons for the first 2 upstream open reading frames reside in contexts suitable for use as initiation codons. Possible mechanisms to explain how ribosomes bypass the upstream open reading frames, including reinitiation, leaky scanning, and IRES activity, were inconsistent with the data. The data are most consistent with a model in which ribosomes bypass or shunt segments of the 5’ leader as they move from the 5’ end of the mRNA to the initiation codon. However, the degree of shunting varied in different cell lines. We hypothesize that the translation efficiency of the BACE1 initiation codon may be increased in Alzheimer’s disease by molecular mechanisms that enhance shunting or increase the relative accessibility of the codon.

We will continue to probe the mechanisms by which short mRNA cis-acting sequences affect translation initiation, and we will test the notion that accessibility may be a key factor in determining whether or not particular nucleotide sequences are recognized as initiation codons.

Transcriptional Control of Vertebrate Development

R. Meech, T.A. Stevens, F.S. Jones, G.M. Edelman

W e focus on the control of vertebrate development by transcription factors. In many instances, the same factors that regulate tissue differentiation and morphogenesis are also involved in misregulated cell growth and migration during neoplasia and degenerative disease. These phenomena are exemplified by the homeobox factor Barx2, which we have studied in both developmental and neoplastic contexts.

Barx2 is expressed in several tissues during embryonic and postnatal development, including the brain, muscles, cartilage, and branching epithelial structures such as the lacrimal and mammary glands. The factor is also expressed in breast cancer cell lines that have receptors for estrogen.

We used a chromatin immunoprecipitation assay to screen for Barx2-binding sequences and to detect target genes in MCF7 breast cancer cells. Several genes were subsequently validated as regulatory targets by using Barx2 short interfering RNA and overexpression in cells. In addition, we found that many of these genes respond synergistically to Barx2 and estrogen treatment, suggesting a convergence between Barx2 binding and signaling by estrogen receptors. Barx2 also promotes anchorage-independent growth of MCF7 cells, which is an estrogen-dependent behavior. We think that coordinated regulation of specific target genes by Barx2 and estrogen receptors underlie this growth effect, and we are using quantitative gene expression analyses to identify such genes.
Barx2 also promotes invasion of MCF7 cells into the extracellular matrix. Recently, we found that Barx2 regulates the expression of several genes that encode matrix metalloproteases and tissue inhibitors of metalloproteases. The products of these genes are secreted by cells, control the degradation of extracellular matrix, and most likely underlie the effects of Barx2 overexpression on cellular invasion. These studies are rapidly revealing the Barx2-regulated pathway that controls invasion by breast cancer cells. Moreover, regulation of both estrogen-dependent growth and cellular invasion is an essential aspect of the normal development of mammary glands, suggesting that the normal function of Barx2 during development may be misregulated in breast cancer, leading to accelerated cancer progression.

In ongoing studies of Barx2 function in the developing limb, we showed that the factor regulates 2 steps of cartilage development: the condensation of mesenchyme and the subsequent differentiation of chondrocytes. The first step involves the regulation of cell adhesion and extracellular matrix molecules, including the neural cell adhesion molecule and tenascin C. Recent results indicated that the second step involves direct regulation of the major cartilage matrix protein collagen II. We identified a new binding site for Barx2 in an enhancer region of the gene for collagen II that controls cartilage-specific expression of this gene. These studies have also indicated that Barx2 may interact functionally with another major regulator of cartilage differentiation, Sox9.

Together these studies have revealed a common function for Barx2 in mammary epithelial tissue and in limb mesenchyme: the regulation of extracellular matrix secretion and degradation. We are now examining the role of Barx2 in cartilage degradation during inflammatory conditions such as arthritis.

In other studies, we used chromatin immunoprecipitation and bioinformatic analyses to identify targets of 2 important regulators of nervous system development: the proneural transcription factor MASH1/HASH1, which promotes differentiation of neural stem cells into particular types of neurons, and the antineural factor NRSF/REST, which inhibits expression of neuronal genes in nonneuronal cells. Our studies indicate that these factors have many common gene targets and that NRSF may also regulate the expression of proneural factors and thus the timing of neuronal differentiation. These studies are helping to define the regulatory networks that control cellular differentiation in the developing brain.

PUBLICATIONS