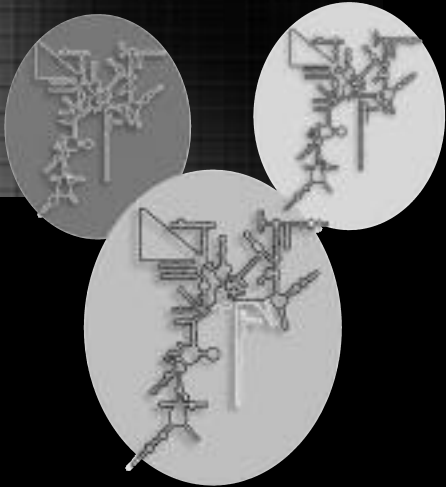


Neurobiology





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**** Appointment completed*

SECTION COVER FOR THE DEPARTMENT OF NEUROBIOLOGY: Schematic representation of a mouse-yeast hybrid 18S rRNA. Yeast expressing the hybrid rRNA were used to demonstrate an mRNA-rRNA base-pairing interaction between a mouse translational enhancer element from the Gtx-homeodomain mRNA and a complementary sequence contained within the 18S rRNA of 40S ribosomal subunits. This work was performed by John Dresios, Stephen Chappell, and Wei Zhou in the laboratory of Vincent P. Mauro, Ph.D. The mouse 18S rRNA is depicted as a black line, and the yeast rRNA is indicated as a white line. The hybrid rRNA contains both mouse and yeast sequences and is depicted as a black-and-white line. The secondary structures were adapted from those obtained on the Comparative RNA Web Site (<http://www.rna.icmb.utexas.edu/>) of Robin Gutell, Ph.D., University of Texas. The artwork was done by Dr. Mauro.



Gerald M. Edelman, M.D., Ph.D.

Chairman's Overview

Members of the Department of Neurobiology continue to focus their efforts on primary cellular processes of development, with emphasis on development of the nervous system in vertebrates. Our earlier work on cell adhesion molecules and the emergence of new technologies prompted us to examine the control of fundamental processes of gene expression in eukaryotic cells. Much of this effort has emphasized the regulation of translation of mRNA into protein, including basic mechanisms of translation and the specific regulation of translation at synapses that occur as the result of particular patterns of synaptic activity. We have also been studying the nature and differentiation of neural stem cells and the basic factors that regulate gene transcription. These various activities are connected by overlapping fundamental principles as well as by the application of common technologies.

Translation in eukaryotes is initiated via 2 mechanisms, cap dependent and internal ribosome entry site (IRES) dependent, which differ in how ribosomes are recruited to the mRNA. In the first mechanism, ribosomes are recruited at the cap structure, a modified nucleotide found at the 5' ends of mRNAs. In the second mechanism, which has been the focus of many of our studies, ribosomes are recruited by IRES elements contained within the mRNA. Differential use of these 2

mechanisms appears to be important in processes such as synaptic plasticity in the brain.

Vince Mauro and his colleagues found that short mRNA sequences can function as IRESs and suggested that some of these sequences affect translation by base pairing to the RNA component of ribosomes, rRNA. Detailed analysis of one of these IRES elements showed that it does form base pairs with rRNA. This pairing was accomplished by altering either the mRNA or the rRNA sequences in a yeast experimental system. Both approaches showed that an intact complementary match was required for activity. These findings are the first conclusive evidence that base pairing is a prominent mechanism in eukaryotic translation. Dr. Mauro and his group have also developed a novel positive-feedback vector-selection system to identify IRES elements from libraries of random nucleotide sequences and have used these individual elements to generate powerful translational enhancers that have applications in biotechnology and gene therapy.

Consolidation of the mechanisms that underlie learning and memory involves an elaborate set of molecular mechanisms that alter the shape and function of the synapse. Some of these changes require protein synthesis, and recent work has revealed a new set of events that link the activity of the synapse to changes in synaptic strength. Apparently, granules containing mRNAs and parts of the translation machinery can be transported to the vicinity of dendritic spines. Synaptic activity can trigger local translation by the elements in the granules, providing specific synaptic proteins at sites of activity.

Peter Vanderklish, Bruce Cunningham, and their colleagues are analyzing factors that regulate local mRNA translation and are determining how the mechanisms that lead to changes in dendritic spines are altered in fragile X syndrome, the most common inherited form of mental retardation. In the mouse model for this syndrome, mice lack the gene for the fragile X mental retardation protein. In these mice, the spines that support the synapses are abnormally long and thin. This abnormality may arise from defects in the regulation of protein synthesis at the synapse. Fragile X mental retardation protein can suppress translation in dendrites, and Dr. Vanderklish and his group have now found that large granules that transport mRNAs and translation machinery to the synapse are reduced in mice that lack this protein.

Working with Dr. Mauro, Dr. Vanderklish also found that the RNA-binding protein RBM3 is present in a subset of dendritic granules and that overexpression of RBM3

can enhance protein synthesis as much as 3-fold. In seeking a mechanism for this increase, these researchers found that RBM3 is associated with the large ribosomal subunit. More critically, overexpression of RBM3 significantly reduced the amount of a microRNA. Because microRNA can influence protein synthesis, this alteration could be a major mechanism by which RBM3 regulates protein expression.

In addition to translational mechanisms, transcriptional control is a major factor in regulating gene expression. Homeodomain transcription factors are critical regulators of gene expression in development. In previous work, we focused our studies on cell adhesion molecules as targets for these transcription factors, which influence the ability of the adhesion molecules to act as regulators of morphogenesis. For more detailed mechanistic studies, Robyn Meech and her colleagues have focused on the homeodomain protein Barx2, which affects a number of developmental processes, particularly in muscle and cartilage development. Moreover, they found that Barx2 affects estrogen-dependent growth and responses to estrogen in breast cancer cell lines.

Overexpression of Barx2 causes the invasion of estrogen receptor-positive breast cancer cells into the extracellular matrix. In cells that lack estrogen receptors, Barx2 expression is lost. These findings and others suggest that coordinate expression of Barx2 and the estrogen receptor α protein occurs. In addition, Dr. Meech has found that Barx2 acts in concert with other factors critical for development, including the homeodomain protein Sox9, the muscle differentiation factor Myo D, and the estrogen receptor.

The vertebrate nervous system is derived from multipotent stem or progenitor cells in the neural tube that divide and differentiate into mature neurons and glial cells. Cell adhesion is a pivotal process in differentiation, and our previous studies indicated that the neural cell adhesion molecule promoted the formation of mature neuronal networks from cultures of neural progenitor cells. In recent studies, Kathryn Crossin and her colleagues have explored the role of energy metabolism in the differentiation of stem cells. Compared with progenitor cells, mature neurons expressed high levels of reactive oxygen species, primarily from mitochondrial metabolism. Dr. Crossin has shown that this property can be used with fluorescence-activated cell sorting to isolate highly enriched fractions of both multipotent stem cells and neurons. Understanding the role of reactive oxygen species in neuronal differentiation and maturation may provide

new means of intervention in development, neurodegeneration, and aging.

The goal of all of these activities is to understand the molecular and cellular events that define and regulate the development of the nervous system. Our efforts have remained focused on the fundamental processes of morphogenesis and neuronal function as well as on a number of related diseases. This strategy is based on the belief that understanding even a single primary process of development can provide the necessary framework for defining key mechanisms that underlie a variety of diseases.

INVESTIGATORS' REPORTS

Cell-Surface and Metabolic Influences on Differentiation of Neural Stem Cells

K.L. Crossin, M. Tsatmali, G.C. Owens, D.B. Edelman, S. Chen, G.M. Edelman

Neural stem cells have great promise for the therapeutic treatment of neurodegenerative diseases and neural trauma. Studies on the use of stem cells for clinical applications, however, point up the need for a better understanding of the fundamental properties of stem and progenitor cells and of agents that influence maturation of the cells into functioning neurons. Recently, we have been exploring the mechanisms of proliferation and differentiation of rat embryonic neural progenitor cells.

Our focus has been the 2 molecular mechanisms that can influence stem cell maturation into neurons. One concern is the role of cell adhesion, particularly the role of the neural cell adhesion molecule (N-CAM). We found that N-CAM added to cultures induced neurogenesis from neural progenitors in vitro and promoted the differentiation of neural progenitors toward neurons that fire spontaneous action potentials. N-CAM was as effective as classical neurotrophins, which are well-known to influence the neuronal phenotype.

Our second area of focus has been how energy metabolism and mitochondrial function contribute to the neuronal phenotype. We found that neurons newly born from cortical progenitor cells have high levels of metabolites called reactive oxygen species (ROS). The finding is surprising because ROS are also associated with aging and cell death. We hypothesized that these species may promote neuronal maturation through a series of biochemical pathways activated by ROS in mitochondria (where the ROS are produced). Support for such positive functions of ROS are emerging from studies of nonneuronal systems. Currently, we are determining whether altering ROS levels in cell culture can influence the ability of progenitor cells to become neurons rather than glial support cells.

The increase in ROS that occurs during neuronal differentiation is due to increased production of ROS by mitochondria, as occurs in normal cellular metabo-

lism, rather than production via plasma membrane oxidases, as occurs in the immune system. We therefore are studying the production of mitochondrial DNA and protein components during normal embryonic development. To date, our results have indicated that the expression of mitochondrial DNA does not follow the same schedule as the expression of certain mitochondrial proteins during development of the cerebral cortex and hippocampus. This finding raises questions about the genesis of respiratory chain complexes and their metabolic activity. We are exploring whether such levels vary in different regions in the brain in adults and in various models of neurodegenerative disease.

In summary, alterations in mitochondrial activity to produce increased ROS accompanies the transition from a neuronal progenitor to a differentiated neuron. Cell contact mediated by N-CAM helps control the maturation of neurons into fully functional, spontaneously active neural networks. Further understanding of both these mechanisms should shed light on neuronal differentiation and may provide a means for intervention in developmental, neurodegenerative, and aging processes.

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

P.W. Vanderklish, A. Aschrafi, A. Atkins, F. Smart, G.M. Edelman

The strength and reliability of synaptic communication between neurons (synaptic efficacy) is not a fixed property. Rather, the ability of synapses to undergo long-term changes in efficacy in response to particular patterns of synaptic activity (synaptic plasticity) is an essential property of neural circuits involved in learning, memory, and various other higher order brain functions. The goal of our research is to define the mechanisms by which long-term forms of activity-dependent synaptic plasticity are consolidated and how these mechanisms are altered in fragile X syndrome (FXS), the most common inherited form of mental retardation.

Three 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, long-term depression (LTD), and synaptic enhancement induced with brain-derived neurotrophic factor. Second, each form of plasticity can be associated with unique changes in the morphology of dendritic spines. Third, changes in efficacy can outlast the half-lives of proteins synthesized during the induction of the changes.

We propose that local translation plays an early and necessary 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. Such regulatory interrelationships are predicted to be unique for each form of plasticity, and those mediating LTD consolidation are proposed to underlie synaptic malfunction in FXS.

In previous work, we found evidence for reciprocal interactions between synaptic translation and structure. We showed that stimulation of metabotropic glutamate receptors (mGluRs) that induce a form of translation-dependent LTD leads to elongation of dendritic spines and that this effect is blocked by a translation inhibitor. In related work, we observed that the translation machinery is reciprocally influenced by determinants of synaptic structure. Treatment of neural preparations with brain-derived neurotrophic factor resulted in translocation of the initiation factor 4E to synaptic mRNA granules. Depolymerization of F-actin and antagonism of integrins blocked this effect.

The changes in spines we observed after stimulation of mGluRs resembled the abnormally long and thin spines seen in FXS.

FXS is caused by the silencing of a gene, *Fmr1*, which encodes a protein (FMRP) that can function as a translational suppressor in dendrites. In mice lacking *Fmr1*, mGluR-induced LTD is enhanced. Because longer, thinner spines contain fewer glutamate receptors, our data support the notion that mGluR-induced translation leads to changes in dendritic spines that express LTD and that this process is not properly limited in FXS.

Our most recent work on the regulation of mRNA granules in the brains of mice lacking *Fmr1* supports the idea that mGluR-induced translation is exaggerated in FXS. Using sucrose gradient fractionation techniques to resolve components of the translation machinery from brain lysates, we found that the levels of heavy mRNA granules were significantly lower in the brains of mice lacking *Fmr1* than in wild-type mice. Parallel

imaging and in vitro studies (and work by others) suggested that granules are composed of translationally silent polysomes, which are "released" into a less dense fraction upon stimulation of translation. Accordingly, we observed that in vivo administration of an antagonist of mGluR5 rapidly increased granules in the brains of mice lacking *Fmr1* to levels matching those in wild-type mice injected with the antagonist. These data indicate that ongoing mGluR activity in brain leads to translation from, and reorganization of, mRNA granules and that FMRP normally limits this process.

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. Antibodies to mRNA-binding proteins found in granules label distinct particles in dendrites, raising the possibility that classes of granules exist that are used differentially during distinct patterns of synaptic activity. Related to this finding, we determined that the RNA-binding motif protein 3 is present in many components of the translation machinery, including a subset of dendritic granules. The protein is distributed in dendrites and affects the activity of a number of translation factors. A major goal for the coming year is to further characterize mRNA granule-based mechanisms of differential translation and their interrelationships with spine structure in wild-type mice and mice that lack *Fmr1*.

Protein-Protein and Protein-RNA Interactions in the Nervous System

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

The nervous system is by far the most complex organization in the body. Accordingly, intricate signals regulate the temporal and spatial development of this system, including signals generated by cell-surface molecules such as cell adhesion molecules and by intracellular protein-protein and protein-RNA interactions.

The neural cell adhesion molecule (N-CAM) is expressed early during development, and its expres-

sion pattern is developmentally regulated. N-CAM mediates cell-cell adhesion through homophilic interactions: N-CAM on one cell binds to N-CAM on an apposing cell. However, defining the mechanism of interaction between 2 cell-surface glycoproteins has been difficult. We used recombinant proteins in a bead-binding assay and transfected and primary cells to clarify the molecular mechanism of N-CAM homophilic binding.

We found that the entire extracellular region of N-CAM mediated bead aggregation; however, the N-terminal immunoglobulin (Ig) domains, Ig1 and Ig2, were essential. These findings were largely in accord with the results of aggregation experiments with transfected L cells or primary chick brain cells. Additionally, maximal binding depended on the integrity of the intramolecular domain-domain interactions throughout the extracellular region. We propose that these interactions maintain the relative orientation of each domain in an optimal configuration for binding.

Thus, it appears that reciprocal interactions between Ig1 and Ig2 are necessary and sufficient for N-CAM homophilic binding, but that maximal binding requires the quaternary structure of the extracellular region defined by intramolecular domain-domain interactions between all 5 Ig domains and the first fibronectin-like repeat.

Adaptation of cells as a consequence of both intracellular and extracellular cues involves changes in protein expression. Regulation of these changes can occur at many levels, including translational regulation. An example of such adaptation is the induction of selected proteins upon exposure of cells to mild hypothermia. One protein whose level in the cell is increased in response to cold shock is the small RNA-binding motif protein 3 (Rbm3). In conjunction with V. Mauro and J. Dresios, Department of Neurobiology, we found that the level of expression of Rbm3 affects overall translation in cells. In stably overexpressing neuronal cell lines, the level of translation increased 3-fold. In addition, overexpression of Rbm3 correlated with a reduction in the levels of microRNAs. Furthermore, we discovered a tight association between Rbm3 and the 60S ribosomal subunit. These findings suggest that Rbm3 plays a significant role in regulating translation.

Currently, we are characterizing endogenously expressed Rbm3 in brain, primary neurons, and selected cell lines. Our results indicate that at least 2 isoforms of Rbm3 are expressed in mouse and that these forms arise from alternative splicing. Rbm3 is found in both the nucleus and the cytoplasm of cells; the precise

distribution depends on both the Rbm3 isoform and the specific cell type. This subcellular distribution is consistent with a role of Rbm3 in mRNA biogenesis. In addition, we found that Rbm3 undergoes extensive posttranslational modifications. Under basal conditions, a proportion of the protein is phosphorylated. We are identifying the additional modifications observed under basal and stimulated conditions; our goal is to determine how such modifications functionally affect Rbm3.

Additional evidence supporting a role for Rbm3 in regulating translation has come from observation of Rbm3 in granules. Granules are protein-RNA complexes that function as vehicles for the transport of selected mRNAs to designated locations within a cell. Granules are thought to be translationally silent and as such provide the machinery whereby protein expression can be restricted to specific cellular locations, such as synapses. The recent identification of many of the protein and mRNA components of granules has provided insight into the assembly and transport of these complexes. We also established that Rbm3 binds to specific mRNAs in primary neurons. This interaction is a direct association between Rbm3 and the RNA, because we were able to show binding between purified recombinant protein and isolated brain mRNA. Currently, we are trying to establish whether the association of Rbm3 with granules is due to protein-protein interactions, protein-RNA interactions, or a combination of both. Our aim is to provide additional insight into the many levels of translational regulation through fundamental studies on the functions of Rbm3.

Translational Regulation of Gene Expression

V.P. Mauro, S.A. Chappell, G.W. Rogers, Jr., W. Zhou, J. Dresios, D.C.Y. Koh, G.M. Edelman

We focus on understanding the mechanisms that underlie the initiation of translation. In eukaryotic cells, mRNAs recruit the translation machinery at a cap structure, a modified nucleotide found at the 5' ends of mRNAs, or at a sequence contained within the mRNA termed an internal ribosome entry site (IRES). Recruitment at the cap structure requires various initiation factors, whereas internal initiation can occur via different mechanisms that vary in their requirements for *trans*-acting factors.

In earlier studies, we showed that some IRESs are composed of shorter functional elements. Currently, we are examining how different IRES elements recruit the translation machinery. To this end, we have developed a powerful new method to facilitate the identification of new IRES elements.

In the new method, a positive feedback mechanism is used to amplify the activities of individual IRES elements. The positive feedback vector encodes a dicistronic mRNA with a reporter gene as the first cistron and the yeast Gal4/VP16 transcription factor as the second cistron. Transcription of this mRNA is driven by a minimal promoter containing 4 copies of the Gal4 upstream activation sequence. In this method, the presence of an IRES in the intercistronic region facilitates the translation of Gal4/VP16, which then binds to the upstream activation sequences and triggers a positive feedback loop that escalates the production of dicistronic mRNA and Gal4/VP16. A corresponding increase in the translation of the first (reporter) cistron is monitored. This reporter also enables isolation of IRES-positive cells. We are using this vector system to identify and analyze new IRES elements.

We are also studying the mechanism by which an IRES module in the 5' leader of the mRNA encoding the *Gtx* homeodomain protein affects the initiation of translation. This IRES module is 9 nucleotides long and is complementary to a segment of the 18S rRNA, which is the RNA component of the small (40S) ribosomal subunit. The results of earlier studies, in which we used binding assays, ultraviolet cross-linking, and functional analyses, suggested that the *Gtx* IRES module enhanced translation by a mechanism that involved base pairing to 18S rRNA. Our most recent results indicated that this IRES element can facilitate the nonlinear movement of ribosomes along the 5' leader of an mRNA.

In addition, findings from studies in yeast provided direct evidence that the mechanism underlying the activity of the *Gtx* IRES module requires base pairing to 18S rRNA. These findings indicated that the activity of this IRES element requires a complementary match within the 18S rRNA. We also showed that the activity of this IRES module can be disrupted by point mutations of the IRES sequence and that activity can be restored by mutations of the 18S rRNA that restore complementarity. These studies provide the first direct evidence of an mRNA-rRNA base-pairing mechanism in eukaryotes.

In other studies, by probing RNA accessibility in living cells, we are testing the notion that accessibility is a key factor in determining whether or not the nucleotide triplet AUG is recognized as an initiation codon. We expect that the results of our studies will provide new insights into the basic mechanisms of the initiation of translation.

Transcriptional Control of Vertebrate Development

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

Vertebrate development is orchestrated by transcription factors that act coordinately to regulate time- and place-dependent gene expression. To identify the molecular mechanisms that underlie this coordination, we focus on the homeodomain transcription factor *Barx2* and its interacting partners. *Barx2*, which was discovered in this laboratory, is expressed in several contexts during embryonic development, including muscle and cartilage of the limbs. We recently found that *Barx2* regulates chondrogenic differentiation and that it cooperates with another major regulator of cartilage differentiation, *Sox9*, to induce expression of the cartilage matrix protein collagen II.

We have now shown that *Barx2* is also an important regulator of muscle development. Using chromatin immunoprecipitation and promoter analysis, we found that *Barx2* regulates muscle-specific gene expression as part of a regulatory complex involving the serum response factor and the myogenic regulatory factor *MyoD*. Moreover, our recent data indicate that *Barx2* has an intriguing dual role in muscle.

In undifferentiated limb mesenchymal cells and myoblast cell lines, *Barx2* transiently promotes myogenic differentiation as indicated by an increase in the early differentiation markers embryonic myosin heavy chain and smooth muscle actin and the appearance of myotubes. However, at a later stage of differentiation, induced expression of *Barx2* causes the disappearance of myotubes and downregulation of myotube markers such as type II myosin heavy chain and myogenin in adults. A similar dedifferentiation effect has been observed previously for the homeodomain protein *Msx1*, suggesting that *Barx2* and *Msx1* may share common targets.

On the basis of these data, we hypothesize that Barx2 promotes both myogenesis in embryos and myotube plasticity in adults. Identifying factors that control the developmental plasticity of muscle is extremely important for the design of strategies to repair damaged or diseased muscle in adults.

Factors such as Barx2 that regulate cellular differentiation and cell-cell interactions are also often involved in neoplastic disease. Indeed, Barx2 influences the progression of ovarian cancer. We found that it is also expressed in estrogen receptor–positive breast cancer cell lines, and it directly regulates expression of the estrogen receptor α gene. Approximately two thirds of human breast cancers have receptors for estrogen and depend on estrogen for cell growth. Thus, whether or not cancer cells have estrogen receptors is an important determinant of disease progression and of responsiveness to antiestrogen treatment.

Using chromatin immunoprecipitation and gene expression analyses, we found that Barx2 can bind to 2 different promoters within the estrogen receptor α gene and differentially regulate the expression of 2 variants of the protein encoded by this gene. These particular variants have different responses to estrogen and thus different effects on cell growth. Consistent with this finding, we also found that overexpression of Barx2 in a breast cancer cell line increased estrogen-dependent growth and modulated transcriptional responses to estrogen.

In addition to promoting growth, overexpression of Barx2 causes invasion of estrogen receptor–positive breast cancer cells into the extracellular matrix. Conversely, expression of Barx2 is lost in invasive ovarian cancer cells that lack estrogen receptors, and its reexpression can inhibit invasion. We also observed that Barx2 expression is lost in highly invasive estrogen receptor–negative breast cancer cell lines. These observations prompt the hypothesis that Barx2 controls invasion differentially in cells positive and negative for estrogen receptors and that Barx2 and the estrogen receptor α gene may coordinately regulate genes that influence invasion.

Overall, these studies shed new light on how the combinatorial activities of diverse families of transcription factors can function in development and influence the onset of disease.

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