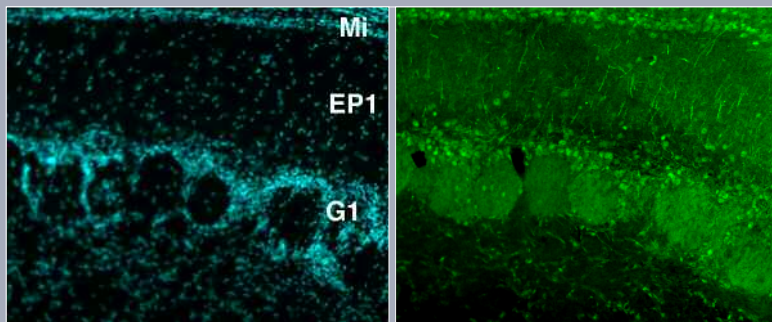


Neurobiology



Immunocytochemical labeling of a coronal section of the olfactory bulb shows high-level expression of RNA-binding motif protein 3 (RBM3). DAPI staining (blue) shows distribution of cell bodies in the mitral cell (Mi), external plexiform (EPI), and glomerular (GI) layers. RBM3 (green) is present in somata within these layers and in neurites within the EPI and interior part of the glomeruli. Figure provided by Julie Pilotte, Ph.D., research associate in the laboratory of Peter W. Vanderklish, Ph.D., assistant professor.



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

Chairman's Overview

Progress in neuroscience has begun to reveal the molecular underpinnings of global brain functions such as learning and memory. That progress has depended on a multilevel analysis of neural function during development. At the most fundamental level, this analysis must revisit basic cellular mechanisms. Recognizing this need, the members of the Department of Neurobiology have directed their efforts to understand how mechanisms of translation, transcription, and metabolic control affect synaptic function. As a result of these efforts, it has become increasingly clear that the fundamental bases of these processes must be reexamined.

Research in a number of laboratories has revealed that a key mechanism governing changes in synaptic strength is activity-related translation of key mRNAs at the synapse itself. Dissecting this process has become a central goal of our scientists' efforts. In pursuing this goal, it became apparent that the fundamental mechanisms of translation in eukaryotic cells had to be reevaluated. Vincent Mauro and his colleagues have carried out a number of studies that have begun to transform our view of the translational regulation of gene expression. Their results have revealed a number of new modes of ribosomal recruitment of mRNA, particularly at the key

regulatory step of initiation of translation. In an elegant set of experiments, they have provided the first direct evidence for base pairing between complementary nucleotide sequences in mRNA and 18S ribosomal RNA. Ribosomal capture of mRNA by this mechanism occurs at an internal ribosome entry site. Currently, Dr. Mauro and his associates are attempting to generalize the result by exploring a variety of mRNAs.

A more commonly studied mode of recruitment is binding of the mRNA cap structure followed by scanning of the 40S small ribosomal subunit from the recruitment site to the initiation codon. Recognizing that this scanning mechanism cannot explain many observations, Dr. Mauro and his colleagues analyzed model RNAs and have provided a basis for a new set of mechanisms. The notion of tethering proposes that the ribosomal subunits reach the initiation codon while attached to the recruitment site (cap or internal ribosome entry site) and then bypass the intervening sequences between these sites and the initiation codon. An alternative mechanism involves clustering, during which ribosomal subunits bind to and detach from various sites on the mRNA. These 2 proposed mechanisms depend on the accessibility of the initiation codon. Accordingly, we have adapted a method to cleave single-stranded RNA sequences, thereby distinguishing regions of accessibility from regions sequestered in double-stranded elements. Dr. Mauro and I have proposed that the ribosome itself is a control element that differentially translates members of the mRNA population. This "ribosome filter" hypothesis has recently been described in a review outlining new supporting evidence.

Parallel to these fundamental studies, Peter Vanderklish and his colleagues have investigated the relationships between local translation at the synapse and synaptic structure. The resulting alteration of structure leads to the consolidation of synaptic plasticity. Their results and the findings of other laboratories indicated that local translation plays a key role in transforming synaptic shape; such changes sustain new states of synaptic efficacy. Previous investigations in our department showed that stimulation of metabotropic glutamate receptors leads to a translation-dependent spine elongation that closely resembles the spine shapes seen in fragile X mental retardation syndrome. In collaboration with J.R. Yates and his group, Department of Cell Biology, Dr. Vanderklish used mass spectrometry techniques to identify many proteins that may account for the synaptic anomalies that occur in a mouse model of fragile X syndrome.

Dr. Vanderklish is also working with Bruce Cunningham to examine the influence of the RNA-binding motif protein 3 (RBM3) on dendritic protein synthesis and its function in other mechanisms that affect protein synthesis. Overexpression of RBM3 enhances translation, and this protein has an important regulatory role in translation in the developing brain. More recent studies suggest that RBM3 has effects on small noncoding RNAs (microRNAs) that can inhibit translation. Drs. Cunningham and Vanderklish and their colleagues have also found that the expression of various forms of RBM3 is regulated through mechanisms controlled by alternative splicing. This research and that on fragile X syndrome indicate the extraordinary range of the components that affect synaptic translation.

In addition to our extensive work on the regulation of gene expression at the level of translation, other studies in our department have focused on critical elements involved in the transcriptional regulation of neural and nonneural development. Robyn Meech and her colleagues have been using a new chromatin immunoprecipitation method to map *in vivo* binding sites for transcription factors. These scientists have focused on the transcription factor REST, which represses the expression of neural proteins in nonneural tissues. These studies have identified new REST targets and uncovered a novel clustering relationship among these targets.

Other developmentally important transcription factors are also being studied, including the homeodomain protein Barx2, which is expressed in a variety of embryonic tissues. In collaboration with H. Makarenkova, Neurosciences Institute, San Diego, California, Dr. Meech and her colleagues have shown that Barx2 regulates muscle-specific gene expression.* Barx2 is found in the stem cell-like population of satellite cells critical for postnatal muscle growth and repair. Mice lacking Barx2 have reduced muscle mass and impaired muscle repair. This transcriptional element may be involved in degenerative muscle disease, a surmise that is being actively studied.

In developmental studies involving differentiation of neural stem cells, Kathryn Crossin has extended her studies of the maturation of neural progenitors to determine the influence of reactive oxygen species produced by mitochondria. She found that high levels of reactive oxygen species in progenitor cells changed the morphology of neurons produced from those cells. Apparently, in addition to their role in cell death, reactive oxygen species in some cells can act as critical regulatory factors. In collaboration with S. Chen, D. Edelman, and G. Owens,

Neurosciences Institute, San Diego, California, Dr. Crossin has studied another mitochondrial function important in neural development: the migration of these organelles to regions of the neuron where mitochondria are needed. Using an imaging system and reagents constructed to specifically label mitochondria, she and her colleagues showed that the neurotransmitter serotonin dramatically enhanced the anterograde transport of mitochondria in axons. They have also analyzed the signaling mechanisms involved in such mitochondrial trafficking. This research promises to shed new light on energy demands during changes in neural states of sleep and waking and in a variety of disease states.

The studies I have summarized have yielded several novel views of fundamental mechanisms of neural development and function. The results promise to give valuable insights into normal brain function and neuronal disease. They reflect the interactive and creative atmosphere supported by our research at Scripps Research.

INVESTIGATORS' REPORTS

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

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

The ability to control the differentiation of neural stem cells into neurons is critical for the therapeutic use of the stem cells. We previously reported that newborn neurons produce higher levels of reactive oxygen species (ROS) than do the progenitor cells from which the neurons are derived. This finding led to the hypothesis that the production of ROS by mitochondria is important for the differentiation and maturation of neural progenitors into newborn neurons.

Although ROS have been associated with cellular stress and cell death via apoptosis, evidence is accumulating for their role as signaling molecules in normal developmental processes in a number of diverse systems. When ROS levels were decreased by overexpression of antioxidant enzymes in cultures of progenitor cells, the total numbers of neurons produced remained the same. However, the types of neurons were altered to favor bipolar cells that expressed the calcium-binding protein calretinin in their nuclei and had a firing rate higher than that of pyramidal-type neurons that expressed calretinin in the cytoplasm and fired few action potentials. Alteration of ROS levels therefore modulates several aspects of neuronal differentiation and can be used to bias a population toward a particular phenotype.

Currently, we are examining the levels of mitochondrial proteins and antioxidant systems to determine how levels of ROS and cellular metabolism are modulated during neuronal development. To this end, we have begun proteomics experiments on highly purified populations of progenitor cells, astrocytes, and neurons. We are also measuring changes in the biochemistry and physiology of a neuroepithelial cell line that differentiates into neurons and may provide an easily perturbable system for further studies.

Another aspect of mitochondrial function particularly critical for neuronal development is the ability of the organelles to migrate to regions of the neuron where energy is needed. We have optimized an imaging sys-

tem to examine mitochondrial motility in real time to search for agents that influence mitochondrial movement into and out of axons. We found recently that the neurotransmitter serotonin, acting through the 5-HT_{1A} receptor, dramatically enhances the anterograde transport of mitochondria in axons. In ongoing studies, we are focusing on the signaling mechanisms that underlie these alterations in mitochondrial trafficking. The results should indicate mechanisms by which mitochondria are transported and how these mechanisms might be impaired in behavioral and disease states.

The results of these and ongoing studies should provide a broad molecular and cellular foundation that could aid in the design of strategies for the expansion and treatment of progenitors for use in clinical applications and also provide a means to alter trafficking in neurons to favor optimal metabolic function.

Structure and Function of RNA-Binding Proteins

B.A. Cunningham, A. Atkins, J. Pilotte, P.W. Vanderklish

Local translation of specific mRNAs at the synapse is critical for the consolidation of synaptic plasticity (lasting changes in the efficacy of neurotransmission). Both mRNAs and the translation machinery are transported from the cell body to the dendrites in granules that move along microtubules. One component of these granules is RNA-binding motif protein 3 (RBM3), a member of a small family of proteins that is upregulated during mild cold shock and other forms of stress. A major goal of our studies is to define the structure and function of RBM3 and other members of this family of proteins.

In earlier studies with V. Mauro, Department of Neurobiology, we showed that overexpression of RBM3 can enhance mRNA translation as much as 3-fold. In structural studies, we identified multiple forms of the protein that are generated by alternative splicing and posttranslational modifications. The protein is expressed in multiple brain regions; the highest levels occur in the cerebellum and the olfactory bulb. In dissociated neurons, RBM3 was detected both in nuclei and in granules within dendrites, and on sucrose gradients, it colocalized with heavy mRNA granules and multiple components of the translational machinery. One of the alternatively spliced forms predominated in dendrites,

although both forms occur in these structures. Overexpression of RBM3 in neuronal cell lines led to increased formation of active polysomes and activation of initiation factors, which, in turn, led to increased translation.

In rats, RBM3 is expressed in the brain as early as embryonic day 14, particularly in differentiating cells in the olfactory bulb, hippocampus, and cerebellum. Expression is highest immediately after birth and begins to decline 2 weeks later. As noted earlier, levels in the brain in adults are low except in the cerebellum and olfactory bulb. Most of the regions with high expression levels correspond to regions of high levels of protein synthesis in the brain, supporting the notion that RBM3 plays a role in regulating translation.

More detailed analyses indicated that RBM3 is usually expressed at highest levels in the nucleus, but in some cell types during development, the high level of nuclear expression shifts to the cytoplasm. In the nucleus, the protein appears in interchromatin granules that contain transcription factors, splicing factors, and RNA-binding proteins. RBM3 does not colocalize with previously defined "splicing speckles," but proteomic analysis done in collaboration with J.R. Yates and colleagues, Department of Cell Biology, indicated an association of RBM3 with proteins involved in splicing. These findings suggest that the protein may regulate mRNA processing as well as mRNA transport and translation. Exon arrays have been used to identify mRNAs whose processing is influenced by RBM3. Preliminary results suggest that the expression of various forms of RBM3 is regulated by mechanisms known to affect at least one class of splicing proteins and have raised the possibility that RBM3 may regulate its own expression profile.

Translational Regulation of Gene Expression

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

In eukaryotes, translation of mRNA into protein begins with recruitment by the mRNA of the translation machinery, which consists of the 40S ribosomal subunit, the initiator methionine-tRNA, and various other factors. This recruitment can occur via the cap structure, which is found at the 5' ends of mRNAs, or at internal sequences contained within some mRNAs. For most mRNAs, the recruitment site is distant from

the nucleotides encoding the protein; thus, before protein synthesis can commence, the translation machinery must reach the initiation codon. These early events are essential for protein synthesis and are key sites of regulation, yet they remain poorly understood. We focus on understanding the mechanisms that underlie these essential initiation events in translation.

RIBOSOMAL RECRUITMENT

Our earlier studies provided the first direct evidence for a mechanism of ribosomal recruitment in eukaryotes that involved base pairing between complementary nucleotides in mRNA and 18S rRNA, which is the RNA component of the 40S ribosomal subunit. The mRNA element in these earlier studies was isolated from the *Gtx* homeodomain mRNA. We showed that this element base pairs with 18S rRNA in the platform of the 40S ribosomal subunit and facilitates initiation of translation. Currently, we are investigating the extent to which this particular base-pairing interaction affects the translation of other mRNAs; we are blocking the interaction in various ways and assessing the effects on the proteome. Preliminary results indicate that this binding site specifically affects the expression of a subset of proteins. In addition to the *Gtx*-binding site, we have identified other putative mRNA-binding sites in the 18S rRNA and are now examining these binding sites and their physiologic relevance.

REACHING THE INITIATION CODON

A generally held model of how ribosomal subunits reach the initiation codon is that they scan from the recruitment site to the initiation codon. Ribosomal scanning is suggested to be linear, that is, each nucleotide is inspected until the initiation codon is encountered, at which point the initiator tRNA base pairs to the initiation codon and scanning stops. However, this model cannot explain various observations reported in the literature and our own findings, a situation that prompted us to suggest alternative mechanisms of translation initiation.

These alternative mechanisms involve tethering or clustering of ribosomal complexes. The notion of tethering suggests that the ribosomal subunits reach the initiation codon while attached to a fixed point in the mRNA, which may be the cap structure or internal mRNA sequences. The tethered ribosomal subunit effectively bypasses sequences located between the ribosomal recruitment site and the initiation codon. In contrast, clustering is a dynamic process in which ribosomal subunits bind to and detach from various sites in the

mRNA. This reversible binding at various sites is postulated to increase the local concentration of ribosomal subunits, increasing the probability that the initiator tRNA will base pair to an initiation codon in the vicinity.

We tested the feasibility of these ideas in studies with model mRNAs. The results indicated that translation efficiency varied with the distance between the ribosomal recruitment site and the initiation codon. In addition, we found that translation could initiate efficiently at AUG codons located upstream of an internal recruitment site. These results are consistent with the notion of ribosomal tethering at the cap structure and clustering at internal sites.

An important prediction of the ribosomal tethering/clustering models is that the accessibility of the initiation codon is an important factor determining the use of the codon. To test this notion, we are studying the *BACE1* mRNA, which encodes the enzyme β -secretase. This enzyme is overexpressed in Alzheimer's disease without a corresponding increase in *BACE1* mRNA levels, suggesting that the translation efficiency of this mRNA is increased in the disease. Our earlier studies indicated that the translation of this mRNA is affected by factors that alter the use of the *BACE1* initiation codon. To assess whether the altered use of this initiation codon is correlated with the accessibility of this codon, we are probing the accessibility of nucleotides within this mRNA in living cells. We have adapted a lead acetate cleavage method that was first used in bacteria to probe short, highly structured RNAs. Our preliminary data indicate that we can detect the endogenous *BACE1* mRNA. Although many of the nucleotides preceding the initiation codon are highly inaccessible to lead cleavage, the initiation codon itself is highly accessible. In ongoing studies, we will experimentally test our hypotheses about accessibility and use of the initiation codon.

Transcriptional Control of Vertebrate Development

R. Meech, O. Harismendy, K.N. Gonzalez

Our overall goal is to define critical elements involved in the transcriptional regulation of mammalian development. As part of a general approach to understanding transcriptional mechanisms, we recently completed a proof-of-principle study in

which we used a new tool based on chromatin immunoprecipitation to identify transcription factor binding sites *in vivo*. This technique combines preparation of chromatin immunoprecipitation tags and multiplex sequencing. Using this tool, we have mapped binding sites for the essential protein REST, which represses the expression of neural proteins in nonneural tissues. With this analysis, we have identified novel REST targets and uncovered a binding-site clustering phenomena that may be important for the mechanism of REST action. This approach can be applied to future studies of other developmentally important transcription factors.

One such factor is the homeodomain protein Barx2, a transcription factor expressed during embryonic and postnatal development in various tissues, including brain, gut, cartilage, and muscle, and in branching tissues, such as mammary, prostate, and lacrimal glands. We have found that Barx2 links 2 very different developmental systems: skeletal muscle and branching organs. To better understand the role of Barx2 in the development of these tissues, we are using mice that lack the gene for this transcription factor.

We found that Barx2 promotes fusion of embryonic myoblasts into multinucleated myofibers in muscle and that it cooperates with classical muscle regulatory factors such as MyoD to regulate muscle-specific gene expression. Postnatally, Barx2 is found in a quiescent stem cell-like population called satellite cells. These cells are responsible for postnatal muscle growth and for muscle repair. We found that mice lacking the gene for Barx2 have less muscle mass than do wild-type mice and do not fully repair injured muscle, suggesting strongly that Barx2 is important for the normal functioning of satellite cells. These findings raise the possibility that Barx2 is a modulator involved in degenerative muscle disease, a notion that we are currently testing by cross-breeding mice that lack the Barx2 gene with mice that carry the gene *mdx*, which is associated with murine muscular dystrophy.

In branching tissues, Barx2 is expressed in epithelial cells, where it regulates interactions between cells and extracellular matrix. Recently, we found that lack of Barx2 dramatically affects the development of 2 branching organs associated with the eye: the lacrimal gland and the harderian gland. These 2 glands produce tears and other secretions that lubricate and protect the eye. In mice that lack the gene for Barx2, these glands are severely reduced in size or absent, leading to ocular defects associated with a "dry eye" condition.

The failure of these glands to branch and develop normally in these mice appears to be largely due to a misregulation of extracellular matrix remodeling factors such as matrix metalloproteinases. This result echoes previous research in which we found that *Barx2* promoted matrix invasion of breast cancer cells by regulating the expression of matrix metalloproteinases. The role of matrix remodeling in both normal development and disease is an ongoing topic of our studies.

Interrelationships Between Local Translation and Synaptic Structure in Consolidation of Plasticity and Fragile X Syndrome

P.W. Vanderklish, J. Pilotte, B.A. Cunningham, G.M. Edelman

Our goal is to define the mechanisms by which long-term forms of activity-dependent synaptic plasticity (i.e., changes in the efficacy of neurotransmission) are consolidated and to determine how these mechanisms may be altered in mental retardation. 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, long-term depression, and synaptic enhancement induced with brain-derived neurotrophic factor (BDNF). Second, each form of plasticity may be associated with unique morphologic changes in dendritic spines. Studies in our laboratory and in others suggest that local translation plays a role in transforming synaptic shape and that changes in synaptic shape ultimately sustain new states of efficacy. Recently, we have focused on determining how dendritically localized mRNA-binding proteins regulate this process and on characterizing the proteins that are made during synaptic plasticity.

The relationship between translation and synaptic morphology is perhaps best illustrated in the neuro-anatomic phenotype of fragile X syndrome (FXS), the most common monogenetic cause of mental retardation. FXS is caused by the loss of a single mRNA-binding protein, the fragile X mental retardation protein (FMRP), which can act as a translational suppressor in dendrites. The hallmark anatomic correlate of FXS

is the presence of abnormally long and thin dendritic spines. Previously, we showed that stimulation of metabotropic glutamate receptors that induce a form of long-term depression leads to translation-dependent spine elongation, resembling spine shapes seen in FXS. In collaboration with J.R. Yates, Department of Cell Biology, we are using high-throughput mass spectrometry to identify differences in the synaptic protein content of cortical neurons from wild-type mice and from mice in which *Fmr1*, the gene that encodes FMRP, has been silenced. In these studies, we identified many proteins that may account for synaptic abnormalities in FXS, some of which may provide opportunities for pharmacologic therapy. In collaboration with N.S. Desai, Neurosciences Institute, San Diego, California, we are also studying the expression of various forms of synaptic and intrinsic plasticity in the neocortex of mice lacking *Fmr1* to gain a better understanding of how many types of plasticity are disrupted in FXS.

In related work, we are characterizing the influence of the RNA-binding motif protein 3 (RBM3) on the synthesis of dendritic proteins. We found that RBM3, a member of a cold-inducible class of mRNA-binding proteins with a simple structure, has properties that are apparently opposite to those observed for FMRP. Primarily, although RBM3 and FMRP are present in the same dendritic mRNA transport granules, RBM3 enhances translation rather than suppressing it. Our previous results suggested that this effect is mediated by a reduction in small noncoding RNAs, so-called microRNAs, that inhibit translation. More recently, we found that the ability of RBM3 to enhance protein synthesis may also be linked to effects on factors that control initiation of mRNA translation. Currently, we are examining the effects of RBM3 on known microRNAs and signaling mechanisms that control mRNA translation. In addition, we have completed a study on the developmental expression of RBM3 in the brains of rodents. Our findings suggest that RBM3 is an important regulator of translation rates in the developing brain and in regions of the brain in adults characterized by high levels of plasticity.

To determine how local translation contributes to the consolidation of synaptic plasticity, we are also conducting high-throughput analyses of the effects of BDNF on the synaptic proteome. We found that numerous proteins with putative structural functions were upregulated in synaptic fractions from neurons treated with BDNF. Interestingly, many components of the transla-

tion machinery were also upregulated, suggesting that BDNF enhances the translational capacity of synapses to facilitate plasticity. Major goals for the coming year include more detailed studies on specific proteins that are altered in mice lacking *Fmr1* and in response to treatment with BDNF. We will also study the mRNA targets that are affected by RBM3-induced changes in microRNAs.

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