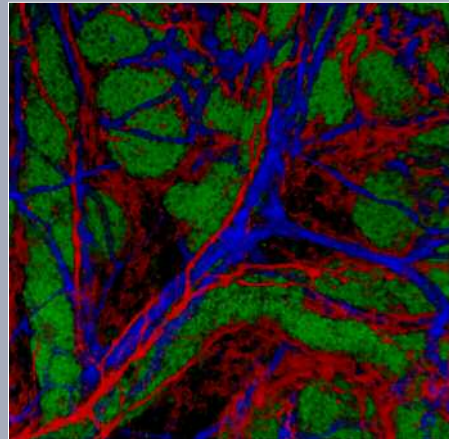
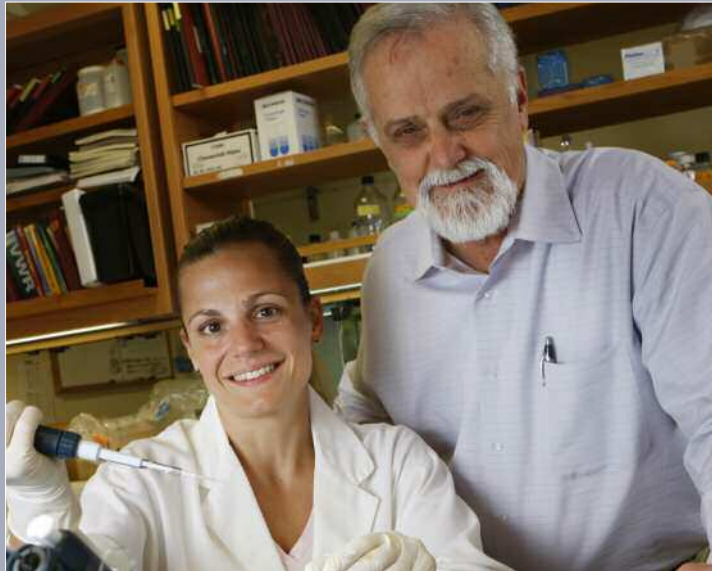


Neurobiology

The lacrimal gland is an example of an organ that develops through branching morphogenesis. Top, A lacrimal gland in explant organ culture consists of a tree-like epithelium surrounded by mesenchyme. Bottom, A confocal image of the lacrimal gland stained with Pax6 antibody to visualize epithelium (green), Pecam antibody (red) to visualize blood vessels, and N-CAM antibody (blue) to detect nerve processes. Images provided by Helen Makarenkova, Ph.D., assistant professor.





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tion shown*

Chairman's Overview

Recent progress in neuroscience has relied in large measure on an increased understanding of the basic processes responsible for global brain functions such as learning and memory. Such progress has required a multidisciplinary approach to the analyses of neuronal development and factors involved in neuronal disease. This effort has in turn revealed that our detailed understanding of many cellular processes must be revisited. Recognizing this need, my colleagues in the Department of Neurobiology have focused their efforts on understanding how basic mechanisms of gene transcription, mRNA translation, and metabolic control influence synaptic modulation and function. As a result of these efforts, new pictures are emerging of the basic cellular processes themselves and of how these processes regulate neuronal function.

Vincent Mauro and his colleagues are continuing to investigate fundamental aspects of translation initiation in eukaryotes. In earlier studies, they showed that base pairing between complementary segments of mRNA and 18S rRNA, the RNA component of the small (40S) ribosomal subunit, can facilitate translation initiation in eukaryotes, much as the Shine-Dalgarno interaction does in bacteria. More recently, they showed that this mechanism also operates in the context of natural mRNAs, including the Gtx homeodomain mRNA and the fibroblast growth factor 2 mRNA, both of which base pair to a sequence contained within helix 26 of the 18S rRNA. The results indicated that this base-pairing interaction could account for approximately 50% or more of the translation mediated by the 5' leaders of these mRNAs.

Dr. Mauro and his colleagues have also extended their analysis of BACE1 mRNA, which encodes the enzyme β -secretase. Increased levels of β -secretase are implicated in the onset of Alzheimer's disease.



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

The increase occurs without a corresponding increase in BACE1 mRNA levels, suggesting that an increase in the translation efficiency of the BACE1 mRNA may occur in the disease. In earlier studies, Dr. Mauro and his colleagues showed that the translation of the BACE1 mRNA occurs via an unusual ribosomal shunting mechanism in which 40S ribosomal subunits are recruited at the 5' end of this mRNA, at a modified nucleotide known as the cap structure, and then bypass intervening sequences, including 4 potential AUG initiation codons, to reach the authentic initiation codon. In more recent studies, they have been able to reconcile contradictory reports on BACE1 translation by showing that various expression systems can alter the translation properties mediated by the BACE1 5' leader. When constructs containing the BACE1 5' leader were transcribed in the nucleus of cells, the upstream sequences were bypassed in the unmutated mRNA. However, when the same constructs were transcribed in the cytoplasm, the upstream AUG codons were used. These studies suggest that under physiologic conditions with transcription occurring in the nucleus, translation of the BACE1 mRNA most likely occurs by a shunting mechanism. The results also highlight a potentially serious limitation associated with the use of cytoplasmic expression systems and with RNA transfections, 2 approaches that are sometimes used for analyses of the mechanisms of translation initiation.

Dr. Mauro and his colleagues are also studying RNA conformation in living cells to assess the importance of this variable in translation initiation. They adapted a lead(II) acetate method from bacteria for use in mammalian cells. The feasibility of the approach was illustrated by using lead acetate to probe the conformation of the RNase P RNA, which has been extensively studied. They have now applied this method to the analysis of the endogenous BACE1 mRNA of rat cells. The results suggest that the accessibility of the initiation codon may be an important variable affecting the site and efficiency of translation initiation. This notion is being tested.

Parallel to these fundamental studies, Peter Vanderklish and his colleagues have investigated the relationships between local translation at the synapse and synaptic structure. Our scientists' results and the findings of researchers in other laboratories indicated that local translation plays a key role in transform-

ing 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 that occur 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 cold-inducible 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, in collaboration with Dr. Yates and L. Liao, Department of Cell Biology, suggest that RBM3 colocalizes with proteins involved in alternative splicing. This research and that on fragile X syndrome indicate the extraordinary range of 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, Helen Makarenkova, Neurosciences Institute, San Diego, California, and their colleagues have studied the homeodomain protein Barx2, which regulates muscle-specific gene expression. Using mice that lack the gene for Barx2, they found that Barx2 influences proliferation and differentiation of muscle satellite cells. The absence of Barx2 in the background of the mdx mouse strain, a model for muscular dystrophy, severely exacerbated the muscle-wasting phenotype. Barx2 is central to the regulation of muscle differentiation and disease; it also appears to play a crucial role in branching morphogenesis in some tissues. Compared with mice with Barx2, mice that lacked Barx2 had dramatically smaller lacrimal glands but completely normal submandibular glands. The connection between Barx2 expression and growth factor signaling may reveal mechanisms and possible therapeutic interventions in dry eye syndrome.

In developmental studies involving differentiation of neural stem cells, Kathryn Crossin has extended her studies that indicated that the reactive oxygen species produced by mitochondria influence the maturation of neural progenitors. She now has shown that the levels of several mitochondrial proteins are increased in neurons and that the mitochondria in neurons are smaller and more numerous than those in neural progenitor cells. 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. In initial studies, they found that the neurotransmitter serotonin dramatically enhanced the anterograde transport of mitochondria in axons. In more recent work, they found that the neurotransmitter dopamine has the opposite effect on mitochondrial trafficking. Thus, neural activity appears to fine-tune mitochondrial transport. 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 described have provided a variety of new views of the molecular and cellular events that underlie normal neuronal development and function. They also promise to provide new insights not only into normal brain function but also into the factors responsible for neuronal disease. The studies reflect a highly interactive approach involving both biochemical and cellular analyses, one that has led to a creative environment for the overall research in our department.

Investigators' Reports

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

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

The ability to control the differentiation of neural stem cells into neurons can provide new insights into neural development and is critical for the therapeutic use of stem cells. We previously reported that alterations in levels of reactive oxygen species modulated several aspects of neuronal differentiation and can be used to bias a population toward a particular phenotype. Our recent studies have shown that the levels of some mitochondrial proteins differ between neurons and neural progenitor cells. More strikingly, electron microscopic studies revealed that the size of mitochondria is much smaller in neurons than in progenitors. Because of this finding, we are focusing on fission and fusion processes in mitochondria and on how these processes influence neuronal fate and differentiation.

Another aspect of mitochondrial function important for neuronal development is mitochondrial migration. As previously reported, we optimized an imaging system to examine mitochondrial motility in real time and found that the neurotransmitter serotonin, acting through the 5-HT_{1A} receptor, dramatically enhances the anterograde transport of mitochondria in axons. More recent studies have shown that the neurotransmitter dopamine has the opposite effect on mitochondrial transport. Thus, neuronal activity appears to fine-tune mitochondrial movement. In ongoing studies, we are investigating the signaling mechanisms that underlie these alterations in mitochondrial trafficking. The results promise to elucidate mechanisms by which mitochondria are transported and how these mechanisms might be impaired in behavioral and disease states.

These studies 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 ways to alter trafficking in neurons to favor optimal metabolic function.

Subcellular Localization and Protein Function

B.A. Cunningham, A.R. Atkins, P.W. Vanderklish

In the cytoplasm, the cold-inducible RNA-binding motif protein 3 (RBM3) plays important roles in regulating mRNA transport and translation. In many cells, however, this protein is more strongly expressed in the nucleus, where its function is unknown. In earlier proteomic studies with L. Liao and J.R. Yates, Department of Cell Biology, we found that RBM3 colocalizes with a subset of proteins from the splicing machinery. Further studies have shown that the protein can influence the splicing of specific mRNAs. Moreover, we have convincing evidence that RBM3 regulates its own expression by splicing an exon with a premature termination codon from its message, preventing degradation of the mRNA via a process known as nonsense-mediated decay.

In other research, we have been characterizing subcellular fractions that may contain assemblies of proteins that play a role in organizing the cytoplasm. Although many macromolecules contain sequences that direct the molecules to specific organelles, how proteins are distributed in the cytoplasm remains a mystery. The number of proteins and the high density of the cytoplasm make it unlikely that simple diffusion could bring proteins to the appropriate collective to carry out intracellular signaling, metabolism, and other functions that require alignment of proteins for specific steps in a cascade of processing. This assumption is especially true for proteins expressed at very low levels.

Our working hypothesis is that cytoskeletal proteins attached to local sites on membranous structures, including the nucleus, endoplasmic reticulum, and plasma membrane, divide the cytoplasm into neighborhoods and that proteins and mRNAs are transported to the appropriate area via specific interactions within this framework. To test this hypothesis, we have been using subcellular fractionation with various detergents and salts in conjunction with electron microscopic examination of the fractions with M. Wood, Core Microscopy, and proteomic analysis with Drs. Liao and Yates. A fraction has been isolated that is highly enriched in a network of cytoskeletal proteins and a small subset of membrane proteins, in accord with our prediction. In the coming year, we will extend these studies by using antibodies to the components of this array of proteins to discern

better the organization and dynamic properties of the complex in living cells.

Molecular Mechanisms of Translation Initiation

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

Translation, a fundamental cellular mechanism, consists of all the processes by which genetic information contained within mRNAs is decoded and expressed as protein. The initiation step encompasses the earliest events in translation. In this step, the mRNA associates with the translation machinery, including the 40S ribosomal subunit and other factors, through recruitment sites in mRNAs. In eukaryotes, recruitment sites include the cap structure, a modified nucleotide found at the 5' ends of mRNAs, and sequences termed internal ribosome entry sites, which are contained within mRNAs. However, because recruitment sites are normally located some distance from the coding sequence, the translation machinery must relocate to the start of the coding sequence (initiation codon) before protein synthesis can begin. These early events in translation are essential for protein synthesis and are important sites of regulation, yet our understanding of these events remains incomplete. Our goal is to understand these critical mechanisms at the molecular level.

RIBOSOMAL RECRUITMENT

In earlier studies, we identified a 9-nucleotide sequence from the *Gtx* homeodomain mRNA that functioned as a ribosomal recruitment site and showed that this sequence could enhance translation by base pairing to complementary nucleotides in 18S rRNA, which is the RNA component of 40S ribosomal subunits. These findings indicated that ribosomal recruitment in eukaryotic mRNAs could occur by base pairing to rRNA. Previously, scientists thought that base pairing between mRNA and rRNA was not used by eukaryotes, although a comparable recruitment mechanism is used by bacteria.

More recently, we investigated the physiologic relevance of mRNA-rRNA base pairing in mammalian cells by using RNA oligonucleotides modified with 2'-O-methyl groups to block *Gtx* sequences in mRNAs or the anti-*Gtx* sequence in 18S rRNA. We looked at 2 mRNAs that contain the *Gtx* element: *Gtx* itself and fibroblast growth factor 2. Studies performed in cell-free lysates

and in transfected cells indicated that translation efficiencies were decreased by more than 50% by oligonucleotides targeting either the rRNA or the mRNA but not by control oligonucleotides. Specificity was demonstrated by showing that the *Gtx* and anti-*Gtx* oligonucleotides did not affect the translation of mRNAs lacking a *Gtx* element and that the effects of these oligonucleotides on the *Gtx* and fibroblast growth factor 2 mRNAs were neutralized by mutation of the *Gtx* elements in the 5' leaders of these mRNAs. Two-dimensional gel electrophoresis revealed that protein expression in cells was generally unaffected by the various oligonucleotides, but that a subset of proteins (up to about 4%) was affected by blocking the base-pairing interaction between *Gtx* and 18S rRNA. We are now using this oligonucleotide-based approach to investigate other putative mRNA-binding sites in 18S rRNA.

REACHING THE INITIATION CODON

We previously hypothesized that ribosomal subunits reach initiation codons while either tethered to the mRNA or clustered near binding sites. Our earlier studies and various observations in the literature strongly support these models. Specific testable predictions can be made on the basis of the ribosomal tethering and clustering hypotheses; many of these predictions differ from those made on the basis of the scanning hypothesis. The scanning hypothesis is a well-established but unsubstantiated model that suggests that ribosomal subunits move linearly along the mRNA, inspecting each nucleotide from the recruitment site to the initiation codon. One goal of our studies is to evaluate the validity of the ribosomal tethering, clustering, and scanning hypotheses.

A key prediction based on ribosomal tethering/clustering but not on scanning is that accessibility of the initiation codon is an important variable that affects recognition of this codon by the translation machinery. We are investigating this notion in living cells by using a lead(II) acetate cleavage method adapted from a method used to study RNA conformation in bacteria. Lead induces cleavage of RNA within single-stranded nucleotides that is essentially sequence nonspecific; it does not effectively induce cleavage of double-stranded RNA.

The feasibility of using this method to assess RNA conformation was demonstrated by probing the endogenous RNase P RNA of rat neuroblastoma cells. The results indicated numerous cleavage sites, which were confirmed in part by using dimethylsulfate to probe accessible adenosine and cytidine nucleotides. In addi-

tion, several cleavage sites were sensitive to competition by magnesium acetate. These results are largely consistent with the conserved features of the RNase P RNA. For comparison, we probed *in vitro* transcribed RNase P RNAs with lead(II) acetate. The results had little resemblance to those obtained in living cells, even when the transcripts were subjected to a denaturation-renaturation protocol. These data validate the use of lead(II) for probing RNAs in mammalian cells and highlight the importance of using such data to evaluate RNA conformations predicted on the basis of other criteria.

We have used the lead-probing method to examine the endogenous BACE1 mRNA, which encodes the enzyme β -secretase. Increased expression of this enzyme is implicated as a possible cause of Alzheimer's disease and occurs without a corresponding increase in BACE1 mRNA levels. This mRNA recruits ribosomes at the 5' cap structure, and its 5' leader contains 4 AUG codons. Although these AUG codons are potential initiation codons, our earlier studies showed that they were not used in some cell types. Probing of the BACE1 mRNA with lead(II) acetate showed that the 5' leader was largely inaccessible, although nucleotides immediately upstream of the initiation codon, the initiation codon itself, and the coding sequences were accessible. These findings support the notion that nucleotide accessibility may help target ribosomes to the BACE1 initiation codon. In addition, different cleavage patterns were obtained when *in vitro* transcribed BACE1 mRNAs were probed, in solution or in a translating lysate, once again highlighting the limitations of such nonphysiologic approaches. We expect that using this approach with other mRNAs will greatly increase our understanding of how ribosomes reach initiation codons during the initiation stage of translation.

DEVELOPMENT OF NEW TECHNOLOGIES

In addition to our basic research on the initiation of translation, we have developed technologies for practical applications, including technologies for increasing protein production in Chinese hamster ovary (CHO) cells, which are the most commonly used cells for the industrial-scale production of protein drugs. We found that the translational enhancers developed in our other studies were relatively inefficient in CHO cells. To obtain enhancers suitable for CHO cells, we used selection methods developed in earlier studies to identify short RNA sequences that function as translational enhancer elements in these cells. By linking various individual elements, we generated a collection of powerful trans-

lational enhancers for CHO cells. Using these elements, we were able to increase production of various recombinant proteins, including reporter proteins, fusion proteins, and human monoclonal antibodies, by approximately 5- to 25-fold.

LONG-TERM GOALS

Our long-term goals are to elucidate the molecular mechanisms of the translation process. We expect that increased understanding of translation will enable us to more fully understand diseases that may involve disruptions of this process, including Alzheimer's disease and many cancers. Our studies may lead to therapeutic strategies or have other practical applications that involve protein expression.

Transcriptional Control of Vertebrate Development

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

We focus on transcriptional regulation in mammalian development. Key factors in this process are the homeodomain proteins such as Barx2, which is expressed during the development of a wide variety of tissues. We have studied 2 very different morphogenetic areas: the musculoskeletal system and branching epithelial glands. Recently, using mutant mice that lack the gene for Barx2, we found that Barx2 controls muscle growth and repair by regulating activation of a population of muscle stem cells called satellite cells. In primary cell cultures, satellite cells from the mutant mice had reduced proliferation, dysregulation of muscle regulatory factors associated with differentiation, and reduced expression of cell adhesion molecules. We have also found that Barx2 is a modulator of muscle disease; *mdx* dystrophic mice that also carry the Barx2 mutation have marked muscle wasting, which is consistent with a failure to repair chronic damage. The role of Barx2 and its target genes in muscle homeostasis, injury, and disease are ongoing areas of study.

Although Barx2 is expressed in many branching organs, it appears to have an especially important role in the lacrimal gland, which produces tears and other secretions that protect the eye. Mice that lack the gene for Barx2 have smaller and defective glands that result in a "dry eye" condition. We found that Barx2 controls matrix remodeling and growth factor signaling in the developing gland. In ongoing research, we are exploring

the connection between matrix remodeling by Barx2 (and other homeodomain factors) and the influence of Barx2 on signaling by growth factors such as fibroblast growth factors, which bind to matrix molecules.

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

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

Our goal is to define the mechanisms by which *de novo* protein synthesis in dendrites (“local translation”) contributes to the consolidation of activity-dependent synaptic plasticity (i.e., changes in the efficacy of neurotransmission). A fundamental observation that guides our hypotheses is that long-term forms of plasticity at glutamatergic synapses are associated with unique morphologic changes in dendritic spines. Studies in our laboratory and others suggest that such changes require local translation and that the new spine shapes are what ultimately sustain altered states of synaptic efficacy. During the past year, we made significant progress in determining how 2 mRNA-binding proteins found in dendrites regulate synaptic form and function through the effects of the binding proteins on local protein synthesis.

The relationship between translation and synaptic morphology is perhaps best illustrated in the neuroanatomic phenotype of fragile X syndrome (FXS), the most common monogenetic cause of mental retardation and a leading cause of autism. FXS is caused by the loss of a single mRNA-binding protein, the fragile X mental retardation protein (FMRP), which can suppress local translation. The hallmark anatomic correlate of FXS is the presence of abnormally long and thin dendritic spines, which our previous research suggests result from a translation-dependent process that is misregulated in the absence of FMRP.

In collaboration with L. Liao and J.R. Yates, Department of Cell Biology, we used high-throughput mass spectrometry techniques to identify synaptic proteins whose expression is altered in mice in which the gene encoding FMRP, *Fmr1*, has been silenced. We identified

approximately 160 proteins with altered expression in cortical synaptic fractions. Many of these changes may account for the synaptic structural abnormalities in FXS, whereas others offer new clues to functional abnormalities that may occur in the syndrome. Follow-up studies, including collaborative efforts with N.S. Desai, Neurosciences Institute, San Diego, California, are being conducted to characterize how the identified proteins regulate the form and electrophysiologic characteristics of cortical synapses.

Our research on another dendritic mRNA-binding protein, the RNA-binding motif protein 3 (RBM3), has provided new insights into the regulation of local translation in dendrites and the contribution of mRNA-binding proteins to basic morphoregulatory events common to many cell types. Previously, we showed that RBM3, a member of a cold-inducible class of mRNA-binding proteins, has several distinctive properties. Primary among these is a strong stimulatory effect on translation. Recently, we found that RBM3 influences several levels of translational control. One likely mechanism is a reduction in the levels of small noncoding RNAs (microRNAs) that act primarily to inhibit translation. Using microRNA arrays, we found that overexpression of RBM3 reduces the levels of a broad set of known microRNAs. In related work, we have obtained evidence that RBM3 also enhances translation by stabilizing mRNAs with adenine-uridine-rich elements and by regulating components of signaling pathways involved in the initiation of translation.

Studies on the developmental expression of RBM3 in the brain in rodents suggest that one role of RBM3 is to regulate translation events that contribute to plasticity. The highest expression of RBM3 in brain occurs during early postnatal development; thereafter, it remains highly expressed in neural progenitors and in regions characterized by high levels of synaptic plasticity. We have used cell lines to investigate the role of RBM3 in morphologic change and have found that it is involved in an early, translation-dependent step in the morphologic maturation of cells in culture. This process may be mechanistically related to morphoregulatory events *in vivo*, including those that underlie the maturation of neural progenitors and the growth of dendritic spines.

In the coming year, we will focus our efforts on characterizing how specific proteins that are expressed at abnormal levels in the mice in which *Fmr1* has been silenced regulate synaptic form and function. In addition, we will evaluate the roles of select microRNAs

(and their targets) in mediating the morphoregulatory effects of RBM3.

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