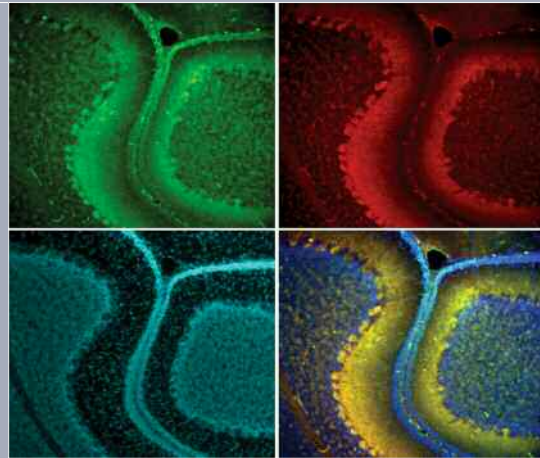


Neurobiology

Rbm3 is highly expressed in the cerebellum. Immunostaining (top left panel, in green) and in situ hybridization (top right panel, in red) for Rbm3 were performed on a sagittal section of 12-day-old rat brain. The bottom left panel (in cyan) shows nuclei stained with 4',6-diamidino-2-phenylindole; the bottom right panel is an overlay of the three panels. Note the high expression of both Rbm3 protein and mRNA in purkinje cell bodies and dendrites. Image provided by Julie Pilotte, Ph.D., and Peter W. Vanderklish, Ph.D.





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Chairman's Report

A vast amount of new information about cellular and neurobiological events has become available as a result of new methodologies, particularly in molecular biology. Integrating these new data into our understanding of central problems of biology continues to be a major challenge. In the past year, members of the Department of Neurobiology have made extensive progress in their studies of the fundamental processes of development, particularly those involved in development of the nervous system and those that regulate synaptic plasticity.

Much can be gained by understanding these mechanisms, particularly those that generate synaptic change to regulate higher-order processes such as learning and memory. The vertebrate nervous system derives from multipotent stem or progenitor cells in the neural tube, which divide and differentiate into mature neurons and glial cells. Kathryn Crossin and her colleagues have provided convincing evidence that reactive oxygen species, molecules usually associated with cell death, provide positive signals to neural stem cells. While not altering the proportion of neurons and glia that arise from the stem cells, these highly reactive molecules regulate the proportion of different neuronal subtypes in development. The results of these studies may provide new mechanisms for regulating the types of neurons that can be

produced from neural progenitors, which in turn will enhance their potential use in stem cell therapies.

Consolidation of the mechanisms that underlie learning and memory involves an elaborate set of molecular events that alters the shape and function of synapses. 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. It appears that 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.

Bruce Cunningham and Peter Vanderklish, along with their colleagues, have extended their studies of the proteins involved in the organization of granules and their transport into dendrites. To these efforts they have added high-throughput proteomics studies in collaboration with Lujian Liao and John Yates. One project focuses on Dr. Vanderklish's analysis of the changes in synaptic components in response to brain-derived neurotrophic factor, which influences a variety of processes in neural development and synaptic plasticity. In another study, these investigators are examining the proteins that interact with the RNA-binding protein RBM3, which is involved in regulating mRNA transport at multiple levels. In earlier studies, they found that RBM3 is present in a subset of dendritic granules and showed that its overexpression can enhance protein synthesis as much as 3-fold.

Dr. Vanderklish has also been studying the biochemical and synaptic events altered in the fragile X mental retardation syndrome. This year he was honored by the Fragile X Research Foundation for his analysis of the molecular and cellular changes in the mouse model for this syndrome. The results of these studies promise to provide new assays for testing drugs to treat the condition.

Although much is to be learned about translation of mRNA at the synapse, there are still considerable gaps in our knowledge of the fundamental events in mRNA translation itself. 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 sequence elements (IRESs) 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 have made major inroads into understanding the events that regulate translation initiation. They have shown convincingly that base pairing between messenger RNA and ribosomal RNA is a critical step in selective initiation of translation of many mRNAs. Moreover, they have found that similar processes are involved in the ability of the translation machinery to “shunt” past structural obstacles, such as hairpin structures in mRNAs, and past upstream start codons (AUGs) that are present in many mRNAs. As these studies gain in breadth, they are beginning to challenge established concepts and are likely to modify current textbook views on the factors that regulate translation initiation.

Transcription of selected genes has long been noted as a key regulator of development. Robyn Meech and her colleagues have developed a method for exhaustive identification of the targets of transcription factors in the genome. Initial experiments are being tested on the transcription factor REST, which plays critical roles in regulating neural-specific genes.

Homeobox genes are particularly important because they control cascades of genes that regulate entire developmental patterns. Dr. Meech and her colleagues are defining the ability of the homeobox protein Barx2 to regulate development in a variety of tissues. Their recent work shows that Barx2 plays multiple roles in muscle development and repair. It also has an intricate pattern of coregulation with the estrogen receptor, a finding that may lead to new insights into the factors that cause breast cancer.

All of these studies open exciting new areas. Moreover, they are gradually merging into what promises to be a series of synergistic interactions among our investigators that will allow them to attack these and other fundamental problems in new ways.

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

The ability to control the differentiation of neural stem cells into neurons is critical for therapeutic use of the stem cells in neurodegenerative disease and neural trauma. During earlier studies on the influence of signaling by cell adhesion molecules on neural stem/progenitor cells, we found another molecular mechanism that appears to be important for the differentiation and maturation of neural progenitors into newborn neurons. This mechanism involves the production of reactive oxygen species (ROS) by mitochondria and the influence of ROS in controlling neuronal development.

Recently, we showed that newborn neurons produce higher levels of ROS than do the progenitor cells from which the neurons are derived. ROS have been associated with cellular stress and cell death by apoptosis. However, evidence is accumulating for a role for ROS signaling in normal developmental processes in several diverse systems. We previously hypothesized that ROS might promote neuronal differentiation or maturation.

We have shown that cells with high levels of ROS occur in embryonic and early postnatal stages of brain development in rats. The cells have normal physiologic properties, indicating that they are healthy neurons. The pattern of expression of ROS is consistent with that of young migratory neurons in the developing cortex. By postnatal day 20, few cells with high levels of ROS remain except in neurogenic regions such as the olfactory bulb and the dentate gyrus of the hippocampus. Thus, it appears that high levels of ROS are a property of newborn neurons.

We explored this possibility further with clonal cultures of cells from the brains of embryonic rats. Cells with low levels of ROS were isolated by using fluorescence-activated cell sorting. At embryonic day 15, such cells from the cortex were multipotent progenitors that differentiated into neurons, astrocytes, and oligodendrocytes when growth factors were removed from the culture. Two types of neurons were produced.

Type I neurons were large pyramidal-like neurons that fired no action potentials or only a single action potential and expressed low levels of the calcium-binding protein calretinin in their processes. These cells accounted for about 70% of the neurons. Type II neurons were smaller bipolar-like neurons with rounded cell bodies that fired repeated action potentials and contained high levels of calretinin in their nuclei. These neurons accounted for about 30% of the neurons in the cultures.

Decreasing the levels of ROS with cell-permeant antioxidants strongly influenced the numbers of the 2 neuronal cell types, although the total numbers of neurons remained the same. Under these conditions, the type II neurons accounted for 80% of the neurons and type I neurons for 20%.

Changes in ROS levels therefore modulate several aspects of neuronal differentiation and could be used in development to bias a population of cells toward a particular phenotype. The results of these and ongoing studies should provide a broad molecular and cellular foundation that may aid in the design of strategies for the expansion and manipulation of progenitors for use in clinical applications.

Structure and Function of RNA-Binding Proteins

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

For some time, we focused on the structure and function of proteins in the nervous system, including cell-surface glycoproteins that mediate cell-cell interactions and proteins involved in synaptic plasticity. Recently, we turned our attention to intracellular proteins that mediate protein-protein and protein-nucleic acid interactions. We have been characterizing RNA-binding motif protein 3 (RBM3), which is induced by mild cold shock and other forms of cellular stress. The protein is part of a small family of proteins characterized by having a single RNA-binding domain and an arginine/glycine-rich domain that is thought to mediate protein-protein interactions. Our structural studies indicate that RBM3 is expressed in multiple forms. At least one of the forms arises by alternative splicing; the others most likely are generated by posttranslational modifications.

In earlier studies, we found that RBM3 is present in granules that are transported from the cell body to

dendrites and that include mRNAs, a variety of proteins, ribosomes, and other parts of the translation machinery. The granules are translationally silent, but their contents can be used for translation at the dendrites after synaptic stimulation. In the past year, in collaboration with V. Mauro, Department of Neurobiology, we found that overexpression of RBM3 can enhance translation as much as 3-fold. The protein was associated with the large ribosomal subunit, but the amounts associated with the ribosome were too small to account for the influence of RBM3 on translation. A more likely possibility is that overexpression of the protein can significantly decrease the size of a fraction that contains micro-RNAs, which influence translation.

To help define the range of functions for RBM3, we examined its expression throughout the brain during development. We found that the levels of expression were highest in early development and decreased with age except in areas of active cell proliferation. The highest levels were in the cerebellum both in embryos and in adults. These results and earlier studies indicated that in addition to being expressed in the cytoplasm, RBM3 is strongly expressed in the nucleus, but the levels of nuclear vs cytoplasmic concentration vary with cell type and the stages of development. Results were similar in studies in cell lines; different cell lines expressed different ratios of nuclear to cytoplasmic levels of RBM3.

RBM3 appears to be present in discrete regions of the nucleus, but its role in the nucleus is at this stage unclear. Currently, we are assessing the role of the various forms of this unusual protein in RNA processing, transport, and translation.

Translational Regulation of Gene Expression

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

We focus on understanding the mechanisms that underlie the initiation of translation. In eukaryotic cells, the first step in translation is recruiting the translation machinery to the mRNA. This recruitment can be mediated by the cap structure, a modified nucleotide found at the 5' ends of mRNAs, or by specific sequences within some mRNAs that act as internal ribosome entry sites. Our earlier studies indicated that such recruitment sites could be less than 10

nucleotides long and revealed that many were complementary to segments of the 18S rRNA, the RNA component of the small (40S) ribosomal subunit.

These findings suggested that some mRNA elements recruit 40S ribosomal subunits directly by base pairing to the subunits. Although mRNA-rRNA base pairing is used by prokaryotic mRNAs to initiate translation, no direct evidence exists for this mechanism in eukaryotes. Our most recent analysis of a short mRNA element from the 5' leader of the mouse *Gtx* homeodomain mRNA now provides this evidence.

To show base pairing between the *Gtx* element and 18S rRNA, we used yeast, taking advantage of the fact that yeast 18S rRNA, which differs from mouse 18S rRNA by approximately 20%, lacks a complementary match to the *Gtx* element. When the *Gtx* element was tested in yeast, in the 5' leader of a reporter mRNA, it did not enhance translation. However, the element did enhance translation in yeast when mouse rRNA sequences containing the complementary match were introduced into yeast ribosomal subunits or when the yeast 18S rRNA was mutated to introduce a complementary match to the *Gtx* element.

We confirmed mRNA-rRNA base pairing by showing that the activity of the *Gtx* element was disrupted by mutations of the 18S rRNA that disrupted complementarity. That activity was restored when the *Gtx* element was mutated to restore complementarity to the 18S rRNA.

In other studies, we showed that the base-pairing interaction between the *Gtx* element and 18S rRNA also facilitates ribosomal shunting, enabling ribosomal subunits to bypass obstacles in the 5' leader of an mRNA, including an upstream initiation codon that resides in a favorable context and a stable RNA hairpin structure. A low level of shunting occurred when multiple *Gtx* elements were present upstream of the obstacles, but shunting was highly efficient when a single *Gtx* element was also present downstream of the obstacle. Control experiments indicated that the high level of activity observed when the *Gtx* elements flanked the obstacle could not be accounted for by the activity of the single downstream element alone. Studies in yeast revealed that the ability of the ribosomal subunits to shunt required base pairing to 18S rRNA. On the basis of these studies, we propose that clustering and/or tethering of the translation machinery in the vicinity of the mRNA increases the likelihood that ribosomes will interact with other accessible recruitment sites in the mRNA,

enhancing the likelihood of direct recognition of the initiation codon via the initiator methionine-tRNA.

If the notion of clustering and/or tethering is correct, initiation of translation should be less efficient as the distance between ribosomal recruitment sites and the initiation codon increases. A second prediction is that translation should begin efficiently regardless of whether ribosomal subunits are recruited from sites located upstream or downstream of the initiation codon. Using model mRNAs with either the 5' cap structure or multiple *Gtx* elements as ribosomal recruitment sites, we recently tested both of these predictions. The results confirmed both predictions. These studies with model mRNAs indicate that initiation of translation can occur efficiently through nonlinear mechanisms, and we suggest that similar mechanisms underlie movement of ribosome subunits during the initiation of translation of natural mRNAs.

Transcriptional Control of Vertebrate Development

R. Meech, O. Harismendy, K.N. Gonzalez, G.M. Edelman

We focus on the basic mechanisms of transcriptional control in mammalian development. One factor of particular interest is the homeodomain transcription factor Barx2, which was discovered in this department. Barx2 is expressed in embryonic cartilage, muscle, and branching tissues such as mammary, prostate, and lacrimal glands. Recently, we showed important roles for Barx2 in the development of several of these tissues.

In muscle cells, Barx2 interacts with the serum response factor and the myogenic regulatory factor MyoD to regulate muscle-specific gene expression. Early in the development of muscle, Barx2 increases cell adhesion and upregulates expression of smooth muscle actin. This change leads to more rapid fusion of the myoblasts into multinucleated myofibers. After birth, Barx2 is down-regulated in myofibers but persists in a quiescent stem cell-like population called satellite cells. Satellite cells are responsible for the repair of muscles in adults. After injury, these cells activate, proliferate, and then differentiate to produce new myofibers.

Our working hypothesis is that Barx2 is involved in muscle repair by promoting the differentiation of myofibers derived from satellite cells. To test this hypothe-

sis, we are examining muscle repair in mice that lack the gene for Barx2.

Interestingly, although Barx2 initially promotes the differentiation of myoblasts into myofibers, its continued expression in myofibers appears to be incompatible with the differentiated state. Thus, ectopic expression of Barx2 in myofibers in culture induces dedifferentiation of the fibers. Dedifferentiation is an important pathway for muscle repair in amphibians. In these animals, muscle damage induces myofibers to dedifferentiate into myoblasts that proliferate and then redifferentiate into new myofibers. However, the importance of this process in mammals remains unclear. We are investigating whether Barx2-induced dedifferentiation is involved in the repair of muscle in mice.

To better understand how transcription factors such as Barx2 control cellular behaviors, we have been developing a method to comprehensively identify transcription factor binding sites by using chromatin immunoprecipitation. After completion of proof-of-principle studies, we will use this method to examine the targets that are bound and regulated by Barx2 at different stages of muscle development and during repair.

Overall, these studies may provide new avenues for treatment of muscle degenerative disease and muscle injury.

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

P.W. Vanderklish, J. Pilotte, G.M. Edelman

The strength and reliability of synaptic communication between neurons (synaptic efficacy) are not fixed properties. 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 other higher order brain functions. The goal of our research is to define the mechanisms by which changes in efficacy 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 by brain-derived neurotrophic factor. Second, changes in efficacy can outlast the half-lives of new proteins synthesized during induction of the changes. Third, each form of plasticity can be associated with unique changes in the morphology of dendritic spines.

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 placement of new proteins that determine synaptic efficacy. Such regulatory interrelationships are predicted to be unique for each form of plasticity, and defects in specific aspects of these consolidation mechanisms are responsible for synaptic abnormalities in FXS.

Previously, we found that stimulation of metabotropic glutamate receptors (mGluRs), which induces a translation-dependent form of LTD, led to a translation-dependent elongation of dendritic spines. The new spines resembled the abnormally long and thin spines that occur in FXS and suggested that this hallmark abnormality of FXS might be the result of an altered LTD consolidation process.

FXS is caused by the silencing of a single gene, *Fmr1*, which encodes a protein (FMRP) that can act as a suppressor of translation in dendrites. In wild-type animals, stimulation of mGluRs leads to synthesis of FMRP, presumably to limit translation by negative feedback. In mice lacking *Fmr1*, mGluR-induced LTD is enhanced. Because longer, thinner spines have fewer glutamate receptors, our data suggest that mGluR-induced translation leads to changes in the shape of spines that express LTD and that this process is exaggerated in FXS.

The preceding observations have led to the proposal that exaggerated mGluR-induced translation leads to changes in synaptic plasticity and shape that are the proximal causes of the signs and symptoms of FXS. This theory, known as the "mGluR theory," has found broad support, including our observation that translational activation of mRNA granules by mGluRs is enhanced in mice lacking *Fmr1*.

Our current research on FXS includes studies of forms of synaptic plasticity that are elicited according to the relative timing of presynaptic and postsynaptic action potentials. Termed spike timing-dependent plas-

ticity, these changes are of interest because they are important in cortical development and function. We discovered that potentiation induced by spike timing-dependent plasticity is absent in the cortex of mice lacking *Fmr1*. Depression elicited by spike timing was normal, as was a form of mGluR-dependent plasticity of intrinsic neuronal properties. However, neither of these forms of plasticity required translation, and the results are thus consistent with the mGluR theory.

To identify protein changes that produce synaptic abnormalities in FXS, in collaboration with L. Liao and J. Yates, Department of Cell Biology, we are examining the "synaptic proteome" of mice lacking *Fmr1*. This analysis has revealed a large set of proteins that are increased or decreased relative to those in wild-type mice. Many of the proteins may represent novel mechanisms whereby lack of FMRP alters synapses.

We are also conducting proteomic analyses of synapses to determine if translation is regulated differently in distinct forms of plasticity. We observed that brain-derived neurotrophic factor upregulates protein synthesis broadly, and we are comparing these effects with those stimulated by receptors that induce long-term potentiation and LTD. In addition, we are comparing the activities of FMRP and a protein that we recently showed enhances protein synthesis, RNA-binding motif protein 3. In collaborative studies with B. Cunningham, Department of Neurobiology, we are studying how the protein affects translation and how it is regulated and expressed in brain.

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