SHORT COURSE II

Posttranscriptional Regulation in Nervous System Development and Plasticity

Organized by Kenneth S. Kosik, MD
and Sourav Banerjee, PhD

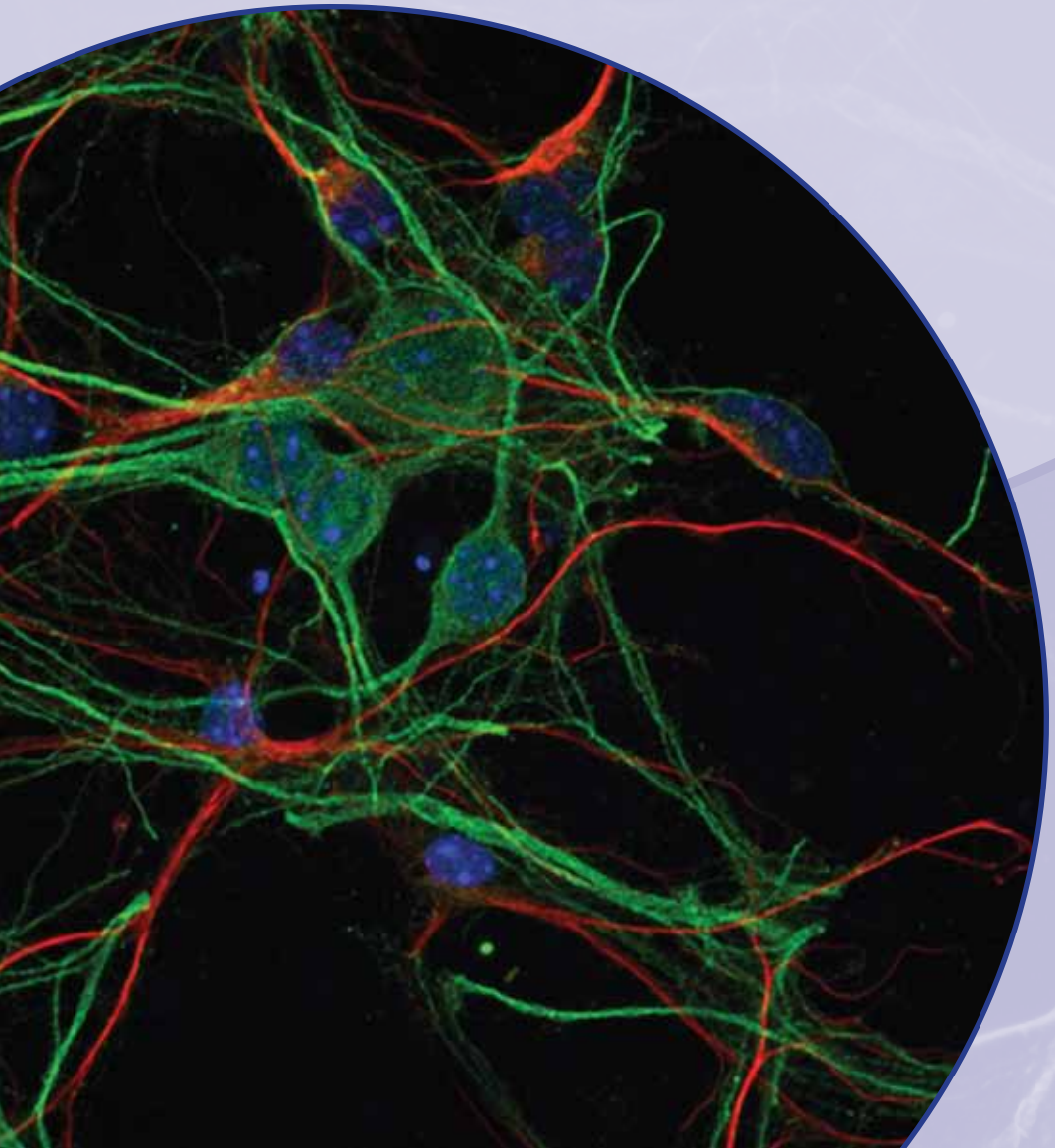

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Table of Contents

Introduction

The emergence of function from a set of molecular parts represents a core challenge in neurobiology. Databases are rapidly filling up with compendia of "omics" information (lists of genes, transcripts, proteins, etc.). Collecting these numerous individual units and using "systems" approaches to deduce function is gradually uncovering the links between the hierarchical levels of biological organization. One system that promises to be particularly informative is the microRNA (miRNA) system of ~21 nucleotide noncoding transcripts. The ~1000 different miRNAs in the mammalian genome form a cytoplasmic layer of posttranscriptional control that is comparable in complexity with the transcriptional control system in the nucleus. Because each miRNA targets many mRNAs, which are often functionally related, miRNA target sets are capable of revealing functional networks of transcripts. Another approach to neuronal systems is taking advantage of the dendrite as distinct cellular compartment. By experimentally capturing proteins and transcripts that are restricted in their localization to the dendrite, one attempts to infer function, particularly functions related to plasticity. Combining miRNA biology with the study of local molecular and physiological activity at the synapse has been a highly productive research vein. However, miRNA biology has opened a much broader window onto the nervous system, and where this system of small RNAs has been most revealing is in nervous system development.

Therefore, using a diversity of model systems, this short course will highlight RNA-mediated regulatory mechanisms involved in fine-tuning the growth and targeting of neuronal dendrites, synapse development, and synaptic plasticity, as well as the development of the neuromuscular junction and lineage decisions in oligodendrocytes. This course will cover many of the most important recent discoveries in this burgeoning area of neurobiology, relevant to both the function and dysfunction of the nervous system. Attendees will be able to discuss their own thoughts informally with speakers in order to shape their current research interest and direction.

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Roles for miRNAs in Timing Developmental Progression Within Nervous Systems

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Introduction

Building an organism requires precisely timed execution of countless developmental events. The nervous system, with its highly connected network of diverse cell types, provides a quintessential example of this complexity. Compounding the developmental complexity of the nervous system, the morphological and functional properties of a given neuron change dramatically over time as it differentiates and later maintains differentiation. Neuron differentiation involves numerous distinctive, highly regulated events, including cell migration, specification of an axon and dendrites, axon guidance, elaboration of dendritic fields, and synaptogenesis. Further, some neurons undergo extensive refinement, including synapse stabilization, synaptic homeostasis, maintenance of axonal and dendritic arbors, and local pruning/remodeling, to name a few. The problem of timing developmental events is further complicated by the necessity for compartment (axon/dendrite) and subcompartment (i.e., apical/basal dendrites)–specific programs. How these events are executed in precise order in a given cell, and how they coordinate the development of surrounding tissue, is largely unknown.

miRNAs as Genetic Switches

Studies of developmental timing have been particularly successful in organisms that progress through well-defined developmental stages, such as *Caenorhabditis elegans*, in which the first microRNAs (miRNAs) were identified as heterochronic mutants (Moss, 2007). Loss-of-function mutations in *lin-4* or *let-7* cause global defects in timing, leading to reiteration of early cell division patterns in later stages; in contrast, gain of function of either miRNA causes precocious division patterns. These results demonstrate that miRNAs can function as genetic switches that globally regulate developmental progression. An unresolved question is whether miRNAs similarly function to regulate developmental timing in neurons. The question is difficult to answer, in part because developmental progression in defined neurons has not been extensively documented *in vivo*. However, transplantation and heterochronic co-culture experiments have shown that competency for particular developmental events is time-delimited within a given neuron, in part because neurons develop over extended periods of time in the presence of, and influenced by, diverse populations of neurons and nonneuronal cells.

From a mechanistic perspective, how do miRNA-based genetic switches function? Conventional wisdom holds that miRNAs function in two main capacities: as rheostats, finely tuning the levels of one or a few primary targets whose dose is critically important, and as molecular switches, which regulate entire developmental programs to toggle between two states, a phenomenon referred to as "bistability." In their function as molecular switches, miRNAs often target transcription factors (TFs), allowing the latter's effects to be amplified (Simon, 2010); the interaction between miRNAs and TFs often involves feedback or feed-forward loops (Tsang et al., 2007; Flynt and Lai, 2008; Simon, 2010). miRNAs that function as genetic switches would seem to fit into the molecular switch class, but the site of action of the miRNA is another important factor to consider. For example, miRNAs functioning nonautonomously could tune the expression of a single gene, such as a growth factor, to trigger a developmental transition in the neuron.

miRNAs in Neuronal Development

Observations that miRNAs are broadly expressed in the nervous system provided the first indications that miRNAs play important roles in neuronal development. Although researchers have not described the complement of miRNAs expressed over developmental time for defined classes of neurons *in vivo*, temporally restricted patterns of expression have been documented for many miRNAs (Kosik and Krichevsky, 2005). Additionally, some miRNAs are enriched in dendrites (Kye et al., 2007); given that axons and dendrites of an individual neuron develop at different times, compartment-specific regulation of developmental timing might be possible. Localization of miRNAs to specific domains of dendrites might facilitate temporally uncoupled development of different dendritic compartments, as with the apical and basal arbors of pyramidal neurons. miRNAs expressed in nonneuronal cells might regulate developmental progression of neurons as well.

In their role as heterochronic genes in *C. elegans*, *lin-4*, and *let-7* family miRNAs function globally (Moss, 2007). However, if miRNAs are to function as timers in the developmental progression of neurons, they must show dynamic and/or localized expression: They may be transiently expressed globally or within a neuron, in surrounding cells, or in cells that neurons contact a given time. How is this dynamic expression regulated? This question is still largely unanswered, but one recent study suggests that miRNA catabolism is likely a particularly important component of this regulation. miRNAs appear to turn over at a higher rate in neurons than in other cell types (Krol et al., 2010), and this increased turnover is dependent on activity and age, at least in cultured neurons. These findings suggest that

NOTES

miRNA catabolism may be particularly adapted for a certain level of maturity or connectivity. However, additional regulatory mechanisms likely influence miRNA levels in neurons because only a subset of miRNAs appear to be subject to this rapid turnover, and only a subset of neurons display this increased rate of miRNA turnover. Target mRNA levels may also influence miRNA levels in neurons, as target mRNAs can stabilize miRNAs in *C. elegans* cell lysates (Chatterjee and Grosshans, 2009). Thus, the interplay among miRNA expression levels, catabolism, and mRNA target levels likely provides the basis for a highly tuned mechanism for timing the progression of neuronal development.

In the spirit of "following the phenotype," this chapter will focus on recent in vivo loss-of-function studies demonstrating that miRNAs function as timers to regulate multiple, distinct aspects of neuronal development. Because several recent reviews have dealt with the roles miRNAs play in dendritic spine morphogenesis, synapse formation, and homeostasis (Schratt, 2009), I will largely omit these topics from my discussion. Additionally, although this chapter focuses on the roles of miRNAs in neuron morphogenesis, it seems likely that miRNAs may regulate transitions in functional properties of neurons, such as changes in ion-channel expression (Okaty et al., 2009). The sections that follow will highlight studies showing that miRNAs can function as timers regulating the acquisition of particular neuronal fates. They do so in part by timing cell cycle progression, regulating postmitotic switches in neuronal and glial growth, and regulating the timing of neuronal remodeling.

Timing of Birth Order: Temporally Regulated Transcription Factors

Neurons derived from a common pool of progenitors often adopt distinct cell fates according to their birth order; thus, the timing of progenitor division is tightly regulated (Kao and Lee, 2010). Birth-dating experiments have established that cortical layers are populated in a temporal sequence (Berry and Rogers, 1965): Cortical neurons in a given layer are born at the same time and share many morphological and functional properties, including layer-specific projection patterns (McConnell, 1988; Kao and Lee, 2010). How timing and birth order are determined is still largely unresolved, but studies in *Drosophila* embryonic neuroblasts (NBs) have defined one paradigm: temporally regulated expression of transcription factors.

Drosophila NBs give rise to different types of motor neurons (MNs) in a stereotyped temporal sequence, and these MNs' temporal identity is determined in part by sequential and transient expression of TFs (Brody and Odenwald, 2005). Temporally regulated TFs appear to regulate the temporal identity of some vertebrate neural progenitors as well. For example, *Ikaros*, a mouse ortholog of *Hunchback* (one of the TFs conferring temporal identity on *Drosophila* NBs), is sufficient to drive early-born cell fates in retinal progenitors (Elliott et al., 2008). And during mammalian cerebral corticogenesis, the TF *Foxg1* regulates the competence of neural precursors by suppressing the generation of the earliest born neurons, Cajal-Retzius cells (Hanashima et al., 2004). One critical question about this paradigm is how the timing of TF accumulation is regulated. Developmental studies using the *Xenopus* retina elegantly illustrate how miRNA-mediated regulation of cell cycle length can time TF accumulation in order to influence neuronal type.

Similar to *Drosophila* NBs, retinal progenitor cells generate different types of neurons (ganglion, horizontal, cone, amacrine, rod, and bipolar) over a conserved time order (Livesey and Cepko, 2001), and different homeobox TFs are important for generation of distinct retinal cell types (Decembrini et al., 2006). How is timely expression of these homeobox TFs achieved? Expression of at least three homeobox TFs involved in fate specification (*XOtx5b*, *Xvsx1*, and *XOtx2*) is posttranscriptionally regulated as follows: Each is transcribed in early progenitors but not translated until later stages, the 3′UTR of each is sufficient to direct time-dependent inhibition of translation, and inactivating *Dicer* in retinal precursors mitigates translation inhibition in these TFs (Decembrini et al., 2008). Thus, it can be seen that miRNAs regulate the timely translation of these TFs. These findings raise an additional question: namely, how timing of miRNA function is regulated in progenitor cells. Cell cycle length of retinal progenitors increases over time (Alexiades and Cepko, 1996), so cell cycle length could contribute to the timing mechanism for miRNA function. Indeed, the expression of *XOtx5b*, *Xvsx1*, and *XOtx2* is linked to cell cycle progression and not to absolute time; deregulating cell cycle length prevents timely translation of these TFs and dissociates birth date from cell fate, generating heterochronic phenotypes (Decembrini et al., 2006). Molecularly, four miRNAs that are specifically expressed (by an unknown mechanism) in early progenitors comprise one portion of this timer by blocking the translation of *XOtx2* and *Xvsx1*, which promote bipolar cell fates in late stage precursors. By extension, miRNAs specific to each stage of retinal (and other) precursors could shape neuronal fates.

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Timing of Stage-Specific Neuronal Growth Programs

Postmitotic neurons often display stage-specific developmental programs such as the establishment and subsequent maintenance and refinement of dendritic coverage (Parrish et al., 2007a). The dendrites of some neurons provide complete, nonredundant coverage of the receptive field, a phenomenon referred to as "dendritic tiling," which is most likely a way to ensure unambiguous representation of the receptive field (Grueber and Sagasti, 2010). In vertebrates and insects, sensory neurons establish tiling of their receptive field early in development and subsequently maintain it, doing so even as the animal grows in size (Bloomfield and Hitchcock, 1991; Parrish et al., 2007a). In the context of development, this presents two very different growth paradigms:

- Initially, dendrites must outpace the growth of their substrate in order to establish coverage of their territory; and
- Subsequently, dendrite growth must be synchronized with substrate growth to maintain tiling.

Drosophila class IV dendrite arborization neurons illustrate this twofold process. They first tile the larval body wall, establishing tiling at approximately the first/second larval instar transition. They subsequently maintain tiling by growing in precise proportion to their substrate: the underlying body wall epithelium (Parrish et al., 2009). This scaling growth of dendrites is manifest in other sensory neurons as well, including those that do not tile, as well as in insect and mouse MNs (Truman and Reiss, 1988; Li et al., 2005), suggesting that it might be a broadly utilized mode of late-stage dendrite growth. As evidence for this theory, loss-of-function mutations in the miRNA *bantam* cause deregulated late-stage dendrite growth of sensory neurons without affecting axon development or early stages of dendrite development. By contrast, *bantam* activity is both necessary and sufficient in the substrate, epithelial cells to trigger the developmental switch from expansive to scaling growth in sensory neurons.

Several lines of evidence point to this transition as being a bona fide developmental step:

1. The structural plasticity of dendrites changes drastically during this transition (Parrish et al., 2009);
2. Distinct genetic pathways regulate the early-growth and late-growth phases of these dendrites (Emoto et al., 2006; Parrish et al., 2007b); and
3. Sensory neurons undergo extensive changes in gene expression patterns at the time of this transition (C. Kim and J. Parrish, unpublished observations).

Notably, in *bantam* mutants, these dendrites inappropriately retain high levels of structural plasticity and other early larval dendrite growth properties.

How is *bantam* regulating this developmental transition? Although the timing mechanism is unknown, *bantam* activity is significantly upregulated at the first-to-second instar larval transition, so hormonal signals that regulate larval molts could contribute to timing (Parrish et al., 2009). Furthermore, whether *bantam* is transcriptionally induced (similar to the way other miRNAs regulate timing) or posttranscriptionally regulated is unknown. One intriguing finding from these studies is that *bantam* regulates the developmental timing of neurons that switch from expansive to scaling dendrite growth at the first-to-second instar larval transition but not in neurons that undergo growth transitions at other times. These findings suggest that other timers (possibly involving other miRNAs) regulate transitions in neurons developing over different time registers. As for *bantam*'s mechanism of action, clonal analysis suggests that *bantam* likely regulates short-range signals that in turn regulate local dendrite-epithelial interactions. Although many questions remain unanswered about how *bantam* regulates the timing of this developmental transition in sensory neurons, these observations demonstrate that miRNAs can coordinate the growth of neurons and their substrate.

One recent report suggests that, as in the sensory neurons described above, miRNAs can regulate stage-specific growth of glia. Schwann cells (SCs) arise from neural crest cells, migrate to surround groups of peripheral nervous system (PNS) axons, and progressively single out PNS axons in a process known as "radial sorting" (Jessen and Mirsky, 2005; Pereira et al., 2010). Subsequently, an SC attaches to the selected axons and alters its gene expression program, in part by activating the expression of the TF *Krox20*, to facilitate production of myelin. In the absence of *Dicer*, most SCs arrest at the promyelinating stage and fail to form myelin; however, the remaining SCs migrate, sort out, and engage axons, demonstrating that the initial stages of SC development are unaffected. At a molecular level, induction of the master regulator of myelin formation *Krox20* and several myelin proteins was not properly accomplished in *Dicer*$^{-/-}$ SCs. The timing of this transition seems to imply that

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axon-derived (perhaps adhesion-based) signaling might be involved in the miRNA switching on of SC growth.

miRNAs as Regulators of Neuronal Remodeling

Following the development of axonal/dendritic arbors, many neurons refine their arbors. One of the most striking examples of this remodeling occurs during insect metamorphosis, when larval-specific processes are pruned away and subsequently replaced by adult-specific processes. Metamorphosis represents a global developmental transition during which most tissues undergo extensive remodeling. Therefore, perhaps it should come as no surprise that the *Drosophila* homologs of the C. *elegans* heterochronic miRNAs *let-7* and *lin-4* (*let-7* and *mir-125* in *Drosophila*) are required for proper timing of metamorphic events.

The expression of *let-7* complex (*let-7*C) miRNAs (*mir-100*, *let-7*, and *mir-125*) is upregulated during the first half of metamorphosis in neurons that broadly innervate the adult, including MNs, and in muscle (Sokol et al., 2008). Consistent with the timing of expression, *let-7*C activity appears dispensable for embryonic and larval development (Caygill and Johnston, 2008). On the other hand, l*et-7*C mutants fail to complete the remodeling of the neuromuscular system that normally occurs during metamorphosis: Larval abdominal muscles that should decay during posteclosion maturation fail to completely disappear, adult muscles are smaller than normal, larval-specific neuromuscular junctions (NMJs) are incompletely pruned, and growth of adult-specific NMJs is stunted (Caygill and Johnston, 2008; Sokol et al., 2008). These effects argue that *let-7*C miRNAs regulate the transition from larva to adult in the neuromuscular system.

How does *let-7* regulate developmental progression in the neuromuscular system? The answer to this question has two parts. First, similar to other miRNAs that regulate developmental transitions, *let-7* likely targets a TF, *abrupt* (*ab*), to regulate remodeling of the neuromuscular system (Caygill and Johnston, 2008). As expected for a *let-7* target, Ab protein accumulates in abdominal muscles in the absence of *let-7*, whereas Ab protein is not detectible in MNs of wild-type or *let-7* mutant flies. Although the relative contribution that *let-7* in muscle and MNs makes to the NMJ defects is unknown, reducing the gene dosage of *ab* was sufficient to partially ameliorate the heterochronic phenotypes of *let-7*, *mir-125* mutants. Furthermore, *let-7* overexpression is sufficient to dampen endogenous expression of Ab, albeit in a different tissue. Whether or not Ab levels contribute to the muscle defects of *let-7*C mutants remains to be seen. Altogether, these results suggest that *let-7* regulates developmental timing of the neuromuscular system in *Drosophila*, at least in part, by regulating levels of Ab.

The second component of the answer as to how timing of *let-7* expression is regulated is even less clear at this point. Although *let-7*C miRNAs can be induced by the nuclear hormone ecdysone in *Drosophila* cell culture, the specifics of hormone-dependent expression have been less well defined in vivo. Given that the nuclear hormone receptor DAF12 positively (presence of ligand) and negatively (absence of ligand) regulates *let-7* expression (Bethke et al., 2009) and that *let-7* regulates *daf-12*, providing feedback inhibition of the switch (Hammell et al., 2009), it seems plausible that a hormone-coupled molecular switch might gate *let-7* expression in flies as well.

let-7 and *lin-4* regulate major developmental transitions during C. *elegans* development by regulating stage-specific patterns of cell division. Do they also regulate developmental transitions in postmitotic neurons in C. *elegans*, as in *Drosophila*? Although the question hasn't been directly addressed, one report implies a role for *lin-4* in regulating postmitotic synaptic remodeling in C. *elegans* (Hallam and Jin, 1998). During larval development, six GABAergic MNs remodel their patterns of connectivity during larval development, completely reversing the direction of information flow; *lin-14* is required for this remodeling. In *lin-14* mutants, MNs remodel precociously. Given that *lin-14* is one of the major *lin-4* targets in lineage decisions, including those that give rise to mechanosensory neurons (Mitani et al., 1993), it seems plausible that *lin-4* might function as a developmental switch in this context as well.

Just as miRNAs regulate a late-stage developmental switch in the *Drosophila* neuromuscular system, *miR-206* regulates a late aspect of development in the mammalian neuromuscular system (Williams et al., 2009). At birth, muscle fibers in mice are multiply innervated by different MNs, but spurious inputs are developmentally eliminated; those that are spared are strengthened (Sanes and Lichtman, 2001). Subsequently, injured muscle can be re-innervated, but innervated muscle does

not get multiply innervated. In a mouse model of amyotrophic lateral sclerosis (ALS) (Gurney et al., 1994), expression of the muscle-specific *miR-206* is dramatically upregulated—a consequence of muscle denervation (Williams et al., 2009). Denervation-dependent induction of the TF *MyoD* leads to upregulation of *miR-206* in muscle, where *miR-206* inhibits translation of the histone deacetylase *HDAC4*; this inhibition, in turn, leads to the de-repression of *fibroblast growth factor–binding protein 1* (*FGFBP1*), a secreted factor that potentiates the effects of FGFs during regeneration. Mice that are deficient for *miR-206* form normal neuromuscular synapses during development but fail to regenerate neuromuscular synapses after acute nerve injury. Therefore, in response to environmental signals (denervation), *miR-206* regulates the release of retrograde signals to potentiate a specialized growth program (re-innervation). Similar to some of the examples cited above, *miR-206* is transcriptionally upregulated, providing the basis for timing, and exerts its effect by inhibiting the expression of a transcriptional regulator.

Perspectives

What do these examples tell us? First, that miRNAs can clearly function as switches to regulate developmental progression in the nervous system, including postmitotic developmental transitions. Even within this small number of examples, trends have emerged:

- miRNAs that regulate developmental progression are often transcriptionally regulated, and those functioning nonautonomously have an additional layer of regulation: environmental cues;
- Many miRNAs appear to primarily target TFs, although miRNAs that function nonautonomously likely encounter an additional time lag because they regulate secreted factors; and
- Consistent with the intimate link between miRNAs regulating developmental progression and TFs, recent studies have shown that transcriptional changes accompany functional maturation of some neurons (Tropea et al., 2006; Okaty et al., 2009).

Where do we go from here? A good starting point would be comprehensive identification of the miRNAs expressed by identified neurons over a developmental time course, coupled with characterization of the roles of miRNAs at different developmental time points in identified neurons (perhaps by conditional knockout of *Dicer*). In principle, this would allow us to systematically identify miRNA-regulated developmental transitions and the miRNAs that regulate them.

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The Regulation of Synaptic Form and Function by microRNAs

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Introduction

The diverse behavioral repertoires of animals, from Cnidaria to primates, all depend on the assembly of neural circuits — ensembles of neurons, sensory organs, and effector cells linked through complex networks of connectivity. At the core of these neural communities is the synapse: a cellular junction dedicated to the communication of neural impulses named by Sherrington more than a century ago (Sherrington, 1906). After many decades of exploration at physiological, cellular, developmental, and molecular levels, we have gained an appreciation for the many stages required to form precise synaptic connections, the molecular landscape that supports each step, and the degree to which misregulation of this intricate process may lead to neurological malfunction and disease. Having such a detailed map of synapse development, function, and plasticity also puts us in an excellent position to examine the layers of regulatory mechanisms required to coordinate the appropriate deployment of the molecules and pathways that enable synapses to form and function. Although transcriptional control systems provide an essential means for establishing or modulating patterns of neuronal gene expression germane to synaptic function, the nervous system clearly relies on multiple posttranscriptional mechanisms to regulate its molecular arsenal (Loya et al., 2010). Among the classes of molecule used to regulate protein expression levels in the brain, the microRNA (miRNA) system probably comprises the most versatile type of known translational regulators.

miRNAs and Their Regulatory Potential at the Synapse

miRNA genes are found in every organism across the metazoan phyla (Bartel and Chen, 2004). Discovered just over a decade ago in *Caenorhabditis elegans* (Lee et al., 1993; Pasquinelli et al., 2000), these tiny (~20-24 nucleotide) noncoding RNAs have enjoyed an explosion of enthusiastic investigation in biological processes, ranging from cell and developmental biology to physiology to disease (Du and Zamore, 2005; Bushati and Cohen, 2007). Our understanding of miRNA biosynthesis and miRNA mechanism of action to repress target gene expression is quite mature and focuses on core machinery that is both well defined and well conserved. At the same time, in the field, the strategies with which miRNAs regulate different biological processes are still emerging rapidly.

Roughly speaking, miRNAs can either eliminate target gene expression to achieve "switch"-like regulation or modulate ("tune") target gene expression across an extended range (Bartel and Chen, 2004; Flynt and Lai, 2008). Their ability to induce a stable biological state by switching off target gene activity is very useful in the nervous system. They do so either at early stages of development, when specific states of cell fate or neuronal differentiation must be achieved and maintained, or under later conditions of neural activity, when synaptic form and/or function must change in a stable fashion. However, many aspects of neural morphogenesis and function require scalable mechanisms that can provide incremental and/or reversible change, such as synaptic expansion in response to increased demand or the homeostatic regulation of neuronal excitability (Turrigiano and Nelson, 2000; Davis, 2006).

Studies of cellular signaling in contexts outside of the nervous system show that miRNAs form essential components for several different classes of regulatory feedback systems. In a simple case, miRNA-dependent downregulation of transcriptional repressors can set the threshold of the default "off" or "on" state. This holds true for multiple, highly conserved "canonical" signaling systems, including the WNT, Notch, and Hedgehog pathways (Inui et al., 2010). For example, conserved members of the miR-200 family (miR-8 in fly and miR-200c in mouse) target the transcription factor TCF and thereby modulate the output of the WNT pathway (Kennel et al., 2008). In addition to simply modulating the basal activity of a molecular pathway, miRNAs can act as amplifiers, attenuators, and feedback-loop components that sculpt and refine the active responses of the cell to signaling events and inputs. For example, in the canonical receptor tyrosine kinase (RTK)–RAS/MAP kinase pathway (essential for many conserved growth factors to function), miR-21 targets multiple downstream inhibitory factors (phosphatase and tensin homolog [PTEN] and Sprouty) and thereby enhances the downstream output (Meng et al., 2007; Thum et al., 2008). On the flip side of this pathway, the highly conserved miRNA let-7 negatively regulates the GTPase RAS (Johnson et al., 2005), thereby reducing the output and providing a powerful means by which to "tune" the proliferative potential of a progenitor cell.

In another canonical signaling pathway that regulates patterning and proliferation, miRNA expression shapes the cellular response to transforming growth factor beta (TGF-β) family ligands (Choi et al., 2007; Martello et al., 2007). Remarkably, regulation of the TGF-β ligand Nodal is performed by distinct miRNAs in different vertebrate species. This variation highlights the fact that, despite rapid evolution of sequence matching between miRNAs

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and their target genes, overall themes in miRNA regulation can be well conserved across phyla (Inui et al, 2010). However, some miRNA–target relationships are well conserved across all bilaterian phyla, providing evidence for co-evolutionary selection (Takane et al., 2010).

Finally, miRNA can also form the initial output of a signaling pathway. An example of this effect can be seen in the maturation of miR-21 downstream of the TGF-β and bone morphogenic protein (BMP) effector SMAD (Davis et al., 2008). Although most of the initial research revealing miRNA control over the canonical cell signaling pathways (e.g., WNT, RTK, BMP) has been performed outside of the nervous system (Inui et al., 2010), many aspects of nervous system development and function rely on these same highly conserved building blocks.

Early Stages in Establishing Neuronal Circuits

The role of miRNAs

Although *in vivo* exploration of miRNA functions in the nervous system is still in its infancy, examples already abound of miRNAs regulating almost every step of neural circuit formation. For example, at the initial stages of neurogenesis and patterning in the zebrafish embryo, disruption of miRNA processing by eliminating all Dicer activity in the embryo reveals rather striking defects on overall brain patterning and morphogenesis (Giraldez et al., 2005). Surprisingly, most of these abnormalities are rescued by reintroducing miR-430 family members. This finding suggests that miRNA control over the early stages of neural development is much more limited than one might anticipate, given the large number of predicted miRNA target genes expressed in the CNS. Indeed, in the mouse, mature and newborn neurons display much higher sensitivity to loss of Dicer activity than do neural progenitor cells (De Pietri Tonelli et al., 2008).

One of the key miRNAs in the early phase of defining a neuronal cell fate is the neural-specific miR-124 (Krichevsky et al., 2006; Cheng et al., 2009; Maiorano and Mallamaci, 2010). This miRNA is a negative regulator of the antineural pathway mediated by the phosphatase SCP1 and its partner transcription factor REST/NRSF (Visvanathan et al., 2007). miR-124 is likely to be a very ancient determinant of neuronal cell identity because it is very well conserved across all bilaterian phyla (Takane et al., 2010). There are other highly conserved miRNA regulators of neurogenesis (Coolen and Bally-Cuif, 2009; Li and Jin, 2010), consistent with the overall conservation of miRNAs that is found in species from worms and flies to humans: ~55% of miRNAs are conserved to some degree (Ibáñez-Ventoso et al., 2008).

In spite of the highly conserved nature of miRNAs, the seed sequence logic of miRNA target matching (Bartel, 2009) provides the opportunity for rapid evolutionary change of this regulatory strategy. Because miRNAs often regulate multiple components in a given pathway, their strategy of regulation has expansive potential for causing rapid evolutionary change. Moreover, like transcription factors that directly control the initial expression of an mRNA, different miRNAs can combine to regulate an individual target gene, thus enhancing the versatility of the miRNA system (Peter, 2010).

For the nervous system, whose complexity at the level of cell fate and connectivity has grown to staggering levels across metazoan evolution, this flexibility of the miRNA system provides a powerful means of remodeling the gene expression landscape and building new dimensions of circuitry over time. Because transcriptional mechanisms of patterning CNS connectivity (Bang and Goulding, 1996) and miRNA control of gene networks operate with combinatorial logic (Peter, 2010), the interaction of these two regulatory layers offers tremendous versatility in the deployment of a limited genome.

Axonogenesis

Once the cellular constituents of a circuit have been defined, connections need to be made, often across substantial distances. The formation and guidance of the axon are key to this long-range level of neural connectivity. Although relatively little is known about the mechanisms that link miRNAs to the cellular machinery required for axonogenesis, miRNAs have been identified that promote axon outgrowth (Sayed et al., 2008; Chen et al., 2010). Additional miRNAs are known to be enriched in the distal portion of the axon (Natera-Naranjo et al., 2010), holding promise that novel functions for these miRNA during axonogenesis may soon be discovered. Again, during the phase of axonogenesis, we find that the highly conserved miR-124 is an essential determinant of neural differentiation in diverse species, from the worm to the mouse embryo (Yu et al., 2008; Clark et al., 2010). Interestingly, recent findings have linked miR-124 to signaling downstream of the axon guidance molecule Ephrin-B1 (Arvanitis et al., 2010).

Axon guidance, like early patterning in the nervous system, relies on a limited and highly conserved arsenal of signaling pathways (Dickson, 2002).

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Ephrins were first appreciated in this capacity in the context of developing connections between the retina and its major synaptic partners in the brain. In the retinal system, disruption of the microprocessor component Dicer has recently elucidated a role for miRNA in the guidance of retinal ganglion cell (RGC) axons through their first intermediate choice point at the optic chiasm (Pinter and Hindges, 2010). At the chiasm, RGC growth cones must choose ipsilateral or contralateral paths to the two sides of the brain, depending on their position in the retinal field. Without Dicer activity, more RGCs extend axons along the ipsilateral path, but many axons are simply lost, projecting either to the contralateral retina or off the normal pathway altogether (Pinter and Hindges, 2010). The number of miRNAs normally expressed during different stages of retinal development is quite substantial (Hackler et al., 2010); however, the particular miRNAs that regulate axon guidance are unknown.

Dendritic morphogenesis

Axons meet most of their synaptic contacts on the surface of dendritic processes. Whereas dendrites explore spatial domains that are much more restricted than their axonal counterparts, many of the same signaling mechanisms govern dendrites' formation and maintenance (Jan and Jan, 2010; Lin and Koleske, 2010). To date, most of the insights into miRNA regulation of dendritic morphogenesis have focused on the plasticity required for circuits to respond to changes in neuronal activity (see next section). However, some of these mechanisms will also be relevant to activity-independent phases of dendritic development. Although the number of miRNAs known to regulate dendritic morphogenesis is limited, several of these focus their regulatory attention on the activity of signaling pathways. For example, miR-138 inhibits dendrite growth by repressing the acyl protein thioesterase (APT1) that regulates the palmitoylation state and activity of $G\alpha_{13}$ (Siegel et al., 2009), presumably upstream of the small GTPase RhoA.

Adding to the list of inhibitory influences, miR-375 has been recently shown to antagonize dendritic growth stimulated by brain-derived neurotrophic factor (BDNF) (Abdelmohsen et al., 2010). However, miR-375 appears to act through the regulation of an RNA binding factor (HuD) known to control mRNA stability and translation in the nervous system (Deschenes-Furry et al., 2006). This mechanism of action implies multiple layers of complexity in the regulatory logic. In other words, miRNA regulation of dendritic morphogenesis is not exclusively inhibitory.

After their initial formation, dendrites in a developing organism also face the challenge of growing to keep up with the size of their target tissue, a process known as "scaling." This normal form of developmental plasticity has been shown to require the miRNA *bantam* in *Drosophila* sensory neurons via regulation of the kinase Akt (Parrish et al., 2009). Although one might guess that such control of sensory dendrite morphology would be intrinsic to the sensory neuron, genetic analysis reveals that *bantam* acts nonautonomously in the underlying epithelium. However, the identity of the signals transmitted from epithelium to sensory neuron is still unknown.

Assembly of presynaptic and postsynaptic structures

Like the formation of dendritic branches, the developmental assembly of presynaptic structures relies on communication between neurons and their target cells. At neuromuscular junctions (NMJs) in *Drosophila*, where retrograde signals are known to sculpt developing synapses (Collins and DiAntonio, 2007), the larval morphogenesis of nerve terminals is regulated by postsynaptic activity of miR-8, a member of the highly conserved miR-200 family (Loya et al., 2009). Perhaps surprisingly, this trans-synaptic phenomenon appears to be mediated largely through the repression of an actin-binding protein (Enabled), raising the question of how postsynaptic structure can determine retrograde signals. At later stages of the *Drosophila* life cycle, when NMJs and muscles remodel, coordinated presynaptic and postsynaptic expression of another conserved miRNA, let-7, takes place (Caygill and Johnston, 2008; Sokol et al., 2008). Loss of the fly let-7 Complex (let-7, miR-100, and miR-125) prevents the normal maturation of these NMJs as these animals metamorphose into adults, largely via dysregulation of the muscle transcription factor Abrupt.

Another class of postsynaptic proteins, essential to synapse formation, comprises the spectrum of neurotransmitter receptor families required to interpret synaptic release. At the fly NMJ, glutamate receptors are regulated by at least one miRNA (Karr et al., 2009). In *C. elegans*, miR-1 controls the expression of both the acetylcholine receptors and the muscle transcription factor MEF-2 (Simon et al., 2008). Interestingly, at this cholinergic NMJ, MEF-2 is upstream of an unknown trans-synaptic retrograde signal that appears to control presynaptic release properties. While this miR-1–MEF-2 pathway may be most relevant to synaptic plasticity, it highlights the intricate ongoing conversation between neurons and their synaptic partners.

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Regulation of Synapse Function and Plasticity by miRNAs

Synaptic plasticity is orchestrated by sophisticated gene expression programs. These programs ensure that environmental stimuli are converted into long-lasting alterations in synapse structure and function (Flavell and Greenberg., 2008). These processes underlie the ability of the brain to adapt to changes in the environment and store information (Kandel, 2001).

Molecular screens for identifying miRNAs

Numerous molecular screens have helped identify miRNAs that are modulating synapse plasticity. One such screen used microarray analysis to identify mRNA populations differentially expressed in distal neuronal processes of rodent hippocampal neurons versus cell bodies. This screen identified more than 100 potentially localized mRNAs, 19 of which were confirmed by *in situ* hybridization to be present in the dendrite (Poon et al., 2006). In another screen, laser capture was combined with multiplex real-time PCR (rtPCR) to quantitatively compare miRNAs in the neuritic and somatic compartments of dendrites from cultured rat hippocampal neurons (Kye et al., 2007). Two additional screens successfully used synaptosomes, a biochemical fraction highly enriched for synaptic membranes, to identify miRNAs localized in the synaptodendritic compartment (Lugli et al., 2008; Siegel et al., 2009). Additional studies are needed to further reveal all the miRNAs involved and to give us a more complete understanding of the role miRNAs are playing in synaptic plasticity.

Dendritic arbor complexity and miRNAs

Increases in dendritic arbor complexity have proven to be an important determinant of synaptic number, size, and function. Transient depolarization, or exposure to neurotrophins, is known to promote dendritic arbor morphogenesis (Wong and Ghosh 2002; Matsuzaki, 2004). miR-132 and miR-134 are two miRNAs that have emerged as playing an important role in the activity-regulated, rapid-response changes of dendritic elaboration (Vo et al., 2005; Wayman et al., 2008; Fiore et al., 2009). miR-132 has been shown to be responsible for the observed increase in dendritic complexity in hippocampal neurons via a switch mechanism through which it decreases the Rho Family GTPase-activating protein p250GAP in an activity-dependent manner (Wayman et al., 2008). In agreement with this, overexpression of miR-132 in hippocampal neurons results in stubby and mushroom-shaped spines with an increase in average protrusion width (Edbaurer et al., 2010).

miR-134, identified in hippocampal neurons as a dendritically localized miRNA, functions as a negative regulator of dendritic spine size (Schratt et al., 2006). *In vivo* work in mouse models recently confirmed the negative role miR-134 plays in dendritic arborization of cortical layer V pyramidal neurons (Christensen et al., 2010). miR-134 acts by tuning *Limk1*, a regulator of actin dynamics. Translational repression of *Limk1* can be relieved by exposing it to the neurotrophin BDNF (Schratt et al., 2006). Additionally, activity-dependent dendritic arbor plasticity occurs through the miR-134 regulation of *pumilio2*, an RBP involved in mRNA transport and translational inhibition (Fiore et al., 2009). Neurotransmitter receptors, the postsynaptic gateway to synaptic activity, can act both upstream and downstream of miRNA mechanisms. Transfection of exogenous ds-miR-132 has been shown to induce the upregulation of glutamate receptors (NR2A, NR2B, and GluRI), suggesting that miR-132 has a positive effect on increased postsynaptic protein levels. Researchers have also noted that, in cultured cortical neurons, BDNF causes a significant upregulation of miR-132 (Kawashima et al., 2010).

However, activity regulation by miRNA is not exclusively postsynaptic. For example, in a screen of *Aplysia Californica*, miR-124 was identified as the most abundant and well conserved brain-specific miRNA, even though its expression there is exclusively presynaptic. At these sensory-motor synapses, miR-124 constrains serotonin-induced synaptic facilitation by regulating the transcription factor CAMP response element-binding protein (CREB) (Rajasethupathy et al., 2009).

Another miRNA recently shown to be important in dendritic spine development is miR-125b. miR-125b and miR-132 (as well as several other miRNAs) are associated with fragile X mental retardation protein (FMRP) in mouse brain. miR-125b overexpression resulted in longer, thinner processes of hippocampal neurons. FMRP knockdown was shown to ameliorate the effect of overexpressed miR-125b and miR-132 on spine morphology. It has been proposed that miR-125b negatively regulates its target, NR2A, through the 3′ untranslated region (UTR), along with FMRP and Argonaute 1 (Edbaurer et al., 2010). This finding provides further evidence to link the miRNA pathway with other RNA-binding proteins that control the translation of synaptic mRNA.

Interestingly, a coordinated local translational control point at the synapse was proposed at which the RNA induced silencing complex (RISC) protein MOV10 was rapidly degraded by the proteasome in an NMDA receptor–mediated, activity-dependent manner. When MOV10 was suppressed, the mRNAs α-*CaMKII*, *Limk1*, and the depalmitoylating enzyme Lysophospholipase 1 (*Lypa 1*) then selectively entered the polysome compartment (Banerjee et al., 2009).

Looking Towards the Horizon

The rapid growth in our comprehension of miRNA-mediated regulatory strategies began just a decade ago. In this short time, it has opened a new window into the molecular complexity of nervous system design. While systematic analysis of miRNA genes in *C. elegans* suggests that the number of miRNAs essential to gross development or viability of the captive organism is somewhat limited (Miska et al., 2007; Alvarez-Saavedra and Horvitz, 2010)—despite the rich source of miRNA targets in this genome (Mangone et al., 2010)—the intricate detail of neural circuits and their behavioral outputs represent a largely untested ground for miRNA function. Exploring the vast and subtle territory of neural circuitry will require sophisticated tools to manipulate miRNA gene function *in vivo* and an exhaustive set of anatomical, functional, and behavioral assays.

During the formation of neural circuits, for example, neurons execute highly specific decisions to innervate the small subset of correct synaptic partners. This initial level of synaptic specificity is technically challenging to assess. Moreover, even our primitive catalog of molecules underlying synaptic target recognition suggests that neurons make these important decisions based on combinations of many cellular cues (Lu et al., 2009). Thus, it may not be surprising that we have yet to find miRNA genes required for this dimension of neural circuit formation. Success in this arena may require either very inventive or very laborious screens. Alternatively, because miRNAs often fine-tune molecular pathway function, it may be necessary to search for such activities using more selective, "sensitized" genetic strategies (Brenner et al., 2010).

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Identification of Novel microRNA Regulatory Proteins in Neurons Using RNAi-Based Screening

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Introduction

The proper functioning of the brain relies on the precise connection of billions of neurons. During the processes of learning and memory formation, specific synapses have to be modulated while neighboring contacts need to stay unchanged. This synaptic plasticity requires *de novo* protein synthesis in the participating synaptic compartments (Sutton and Schuman, 2006; Bramham and Wells, 2007). The local translation of preexisting mRNAs in dendrites is one way to ensure tight spatiotemporal regulation of protein expression in a highly synapse-specific manner.

During the past few years, a number of studies have assigned an important role for the regulation of local mRNA translation in neurons to microRNAs (miRNAs), a class of small noncoding RNAs (Bicker and Schratt, 2008). miRNAs exert a repressive effect on gene expression by binding to partially complementary sequences within the 3′ untranslated region (3′UTR) of target mRNAs. This repression leads to an inhibition of productive translation from the respective transcripts (Bartel, 2004). Owing to their mode of action, miRNAs represent an excellent way to regulate gene expression posttranscriptionally in a tight spatial and temporal manner. Consequently, they are involved in a great variety of cellular processes, including differentiation, metabolism, and cell death. In the brain, miRNAs play a crucial role at different stages of neuronal development and maturation (Fiore et al., 2008).

In order to achieve repression of translation, miRNAs recruit a multiprotein complex to the target mRNA: the so-called miRNA-associated RNA-induced-silencing complex (miRISC). Previous studies identified several important regulators of miRNA function, among which are either core components of the miRISC (e.g., Ago, GW182, Rck/p54, MOV10) (Chu and Rana, 2006; Chendrimada et al., 2007; Peters and Meister, 2007; Banerjee et al., 2009) or other regulatory proteins not directly associated with RISC (e.g., Dnd1, HuR) (Bhattacharyya et al., 2006; Kedde et al., 2007). Strikingly, all studies that screened systematically for miRNA regulators were performed in nonneuronal cells, leaving open the possibility that critical neuron-specific regulators of miRISC function remain to be identified. This possibility is particularly intriguing because such regulators could couple synaptic stimulation to miRNA-dependent control of local translation.

In this chapter, we describe a large-scale screening approach for testing the involvement of neuronal RNA-binding proteins (RBPs) in miRISC function. We present evidence that neuronal miRISC function relies not only on some of the RBPs previously identified in nonneuronal systems, but also on additional proteins that could allow miRNA function to adapt to the special needs of this highly specialized cell type to adapt.

Setting Up a Screening Approach for Regulators of miRNA Function in Neurons

In order to identify miRNA regulators in neurons, we set up a large-scale RNA interference (RNAi) screening experiment. We postulated that the knockdown of important effector proteins in neurons should relieve miRNA-mediated translational inhibition. We used the brain-specific miR-134 as a paradigm, since we had recently demonstrated an important role for this miRNA in the regulation of local mRNA translation in neurons. In mature hippocampal neurons, miR-134 localizes to dendrites, where it inhibits the translation of the Lim-domain-containing kinase 1 (LimK1) mRNA, thereby acting as a negative regulator of dendritic spine size (Schratt et al., 2006). The very same miRNA was later shown to regulate dendritic outgrowth by fine-tuning protein levels of the translational repressor Pumilio2 (Pum2) (Fiore et al., 2009).

For the RNAi screening experiment, we decided to analyze the function of miR-134 using a Luciferase reporter assay. We selected a reporter gene containing an miR 134 target 3′UTR downstream of the Luciferase coding sequence. The reporter-transcript we used harbors the 3′UTR of a newly identified miR-134 target gene that, in comparison with the other miR-134 targets (Limk1 and Pum2), is more strongly repressed by miR-134, making this a suitable readout for a large-scale RNAi screen.

The miRISC is formed by a few core components, such as Argonaute proteins and RCK/p54, and a number of associated factors, e.g., the fragile X mental retardation protein (FMRP) and the Vasa intronic gene (VIG) protein (Caudy et al., 2002), all of which are RBPs. In addition, RBPs like Dnd1 and HuR have been shown to interact with the 3′UTR of the target mRNAs, thereby modulating miRNA activity in an indirect way (Bhattacharyya et al., 2006; Kedde et al., 2007).

RBPs appear to be the key players of the miRNA effector machinery. Therefore, to study regulators of miRNA function in neurons, we decided to focus on this class of proteins. Using small interfering RNA (siRNA) technology, we planned to knock

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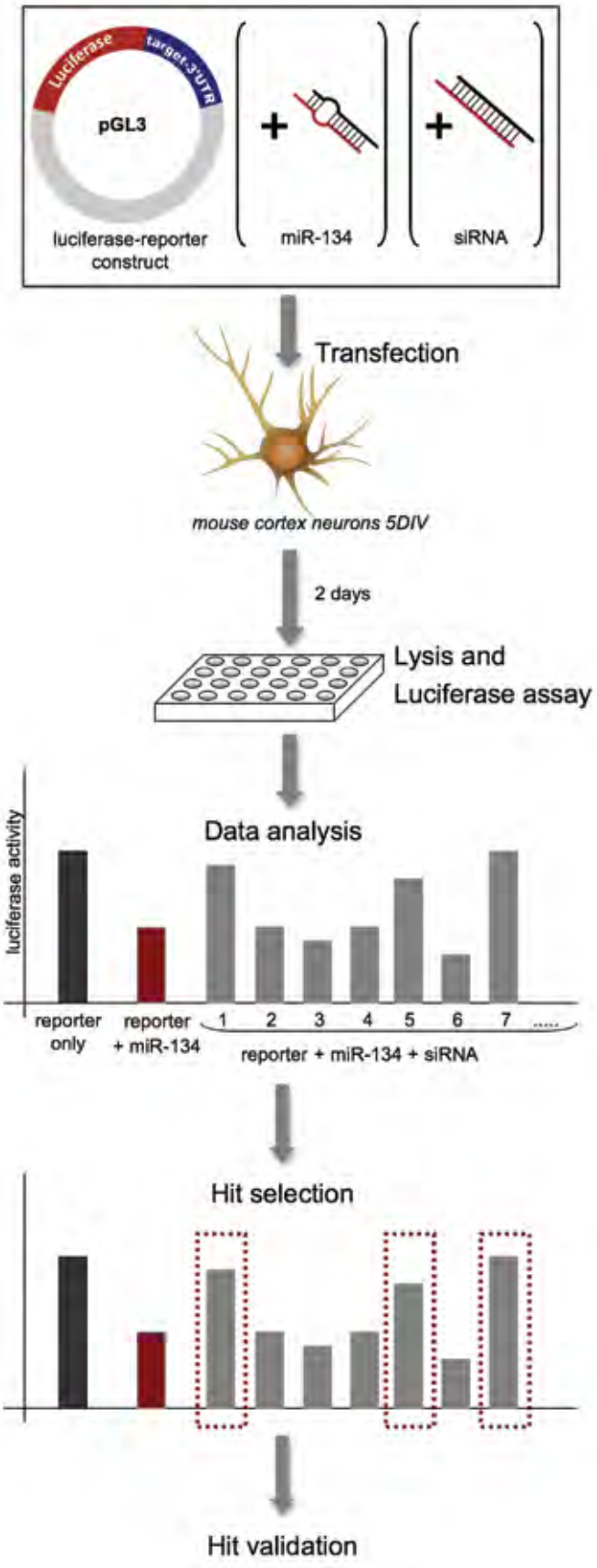

Figure 1. Flowchart of the RNAi screening experiment to identify regulators of miR-134 function.

down each neuronal RBP individually and assess its importance for miRNA-mediated translational repression in neurons using the described Luciferase reporter assay. Based on a previous *in situ* hybridization study (McKee et al., 2005), we listed all RBPs that had been shown to be expressed in postnatal mouse forebrain (~300) and ordered a custom siRNA library with three individual siRNAs for each candidate gene (Ambion, Austin, TX).

In initial experiments, the functionality of our approach was proven by knockdown of the RISC protein GW182 in neuronal cultures, which efficiently interfered with miRNA-134–mediated repression of the Luciferase reporter. For the actual screening experiments, 5 days *in vitro* (DIV) mouse primary cortical neurons were transfected with the miR-134–responsive Luciferase-reporter construct, together with the mature miR-134 duplex and an siRNA targeting one of the RBP genes. Luciferase assays were performed 2 d after transfection. Each condition was transfected in duplicates, and the entire screen was repeated twice.

Hit Validation

miR-134

For hit selection, we applied a threshold where at least two out of three different siRNAs for each targeted gene were able to relieve the miR-134–mediated repression by at least 50% in all three runs. Applying this threshold, the screen identified 10 RBPs that are required for miR-134–mediated repression of the target gene. Among the hits are known components of the miRNA pathway (GW182, RCK/p54) as well as RBPs that have not yet been implicated in miRNA function and therefore might be important specifically in the neuronal system.

To test the efficacy of the siRNAs, we cloned constructs for overexpression of a green fluorescent protein (GFP)–tagged version of the candidate RBPs. Those constructs have been cotransfected, together with the siRNAs, into

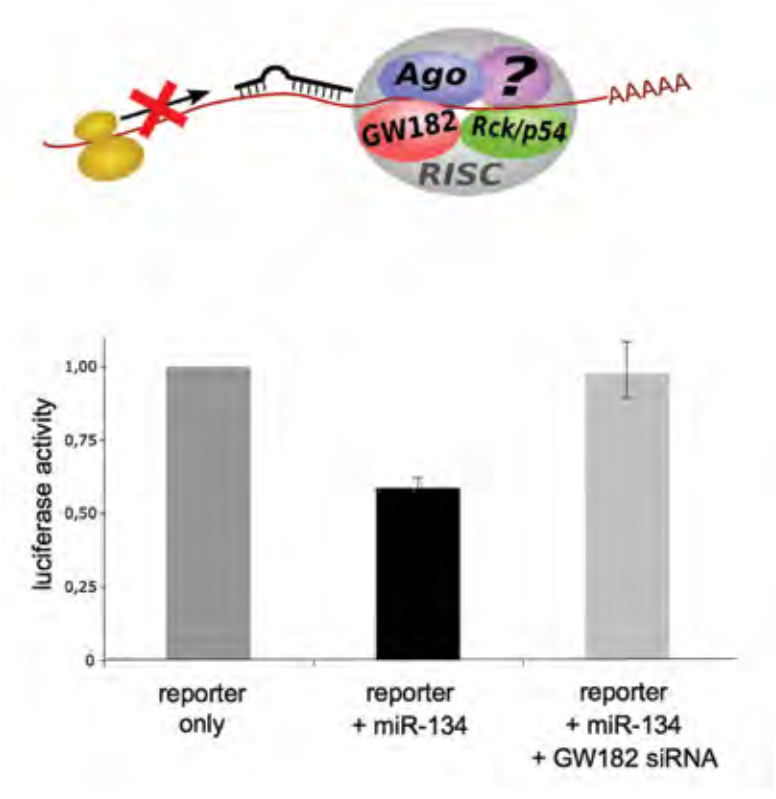

Figure 2. Knockdown of critical RISC proteins leads to the release of miR-134–dependent repression of reporter gene expression. Top: Schematic of the miRNA-associated RISC complex assembled on a target mRNA. RISC components that have been shown to be required for miRNA-mediated repression are indicated. Bottom: Results from a Luciferase reporter assay in cortical neurons using a GW182-siRNA in the context of miR-134 duplex RNA. Bars represent the mean of three independent experiments.

Hek293 cells, and their efficacy was assessed by immunoblotting using a GFP-specific antibody.

Next, we decided to validate the positive hits using several variations of the reporter assay. First, we verified whether the observed effects were indeed the result of impaired miR-134 function or whether the siRNAs affect 3′UTR–dependent translation in a more general fashion. To test this hypothesis, we repeated the Luciferase assay for the identified candidates using a Luciferase reporter construct with a mutated miR-134 binding site that does not show significant repression by miR-134. By doing so, we could discriminate between effects that result from a specific interaction with miR-134 and more general effects on mRNA translation that might arise by reason of either binding motifs for the candidate RBPs in the Luciferase reporter mRNA or direct contacts to the cap structure.

Our results indicated that knockdown of the candidate RBPs leads to an increased expression of the Luciferase reporter not only for the construct harboring the miR-134 binding site but also for the reporter-transcript with the mutated binding site. Therefore, the identified RBPs appeared not only to affect miRNA-134–mediated repression but to play a more general role in terms of translation regulation. This finding is not too surprising, however, because previous studies have shown that the miRISC components GW182, Ago2, and RCK/p54 behave in a similar fashion.

Next, we investigated whether the identified RBPs are general regulators of miRNA function in neurons, or whether some are specific for the studied miR-134–target interaction. To this end, we studied the effect of the candidate siRNAs in Luciferase reporter assays with different combinations of synaptic miRNAs and targets. We tested the two other validated miR-134 targets, LimK1 and Pum2, which allowed us to determine whether the RBP modulates miR-134 function in the context of a specific target gene (i.e., via direct interaction with the 3′UTR) or whether it is able to modulate miR-134 activity in general (i.e., by contributing to the miR-134–specific RISC). We found that the knockdown of our candidate RBPs interfered with miR-134–mediated translational repression independently of the target reporter construct. Thus, we concluded that the identified RBPs seem to play a more general role in miR-134–dependent translational control.

miR-138

Finally, we analyzed whether or not the RBPs also regulate the function of other dendritic miRNAs. The brain-enriched miR-138 is present at synaptic sites and functions, similar to miR-134, as a negative regulator of dendritic spine size. This effect is mediated, at least in part, by translational downregulation of its target, acyl-protein-thioesterase 1 (APT 1) (Siegel et al., 2009). In order to test whether our candidate RBPs are involved in the regulation of miR-138, we tested the respective siRNAs in the context of miR-138–mediated repression of APT 1. For all RBPs tested, the knockdown showed very similar effects

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on miR-138 and miR-134 function. Therefore, we concluded that the identified RBPs likely regulate the function of neuronal miRNAs in a more general way rather than in an miRNA-specific or target-specific manner. This finding, however, does not exclude the possibility that the function of the candidate RBPs may be modified in the context of a specific miRNA–target interaction.

We reasoned that RBPs that are able to regulate miRNA function at the level of the mature miRNA likely reside in the cytoplasm. To gain insight into the subcellular localization of our candidate RBPs, we transfected the described GFP-fusion proteins into hippocampal neurons and assessed the distribution of the GFP-signal using confocal microscopy. The results revealed that the majority of the candidate RBPs showed a pronounced or exclusive signal within the nucleus, matching their described biological function during mRNA splicing or nuclear export.

This observation raises the question: Why were these RBPs required for miRNA function in our RNAi screening setup? Because we transfected mature miRNA duplex, which bypasses processing, we can rule out the option that the nuclear localized RBPs affect miRNA function by simply blocking the first miRNA processing steps in the nucleus. Since we know that most of the candidate RBPs are involved in RNA export or RNA splicing, a possible scenario could be that these RBPs play a significant role in the proper transport or processing of mRNAs encoding important regulator proteins of miRNA function, e.g., Argonaute proteins or GW182. Alternatively, these RBPs might be present in the cytoplasm at very low levels, which we were unable to detect. Additional experiments beyond the scope of this study are needed to discriminate between these possibilities.

Experimental Outlook

Interestingly, a subset of our candidate RBPs was expressed throughout the whole neuron, including the most distal dendritic branches. These candidates are particularly promising because they might play a direct role in the regulation of miRNA function

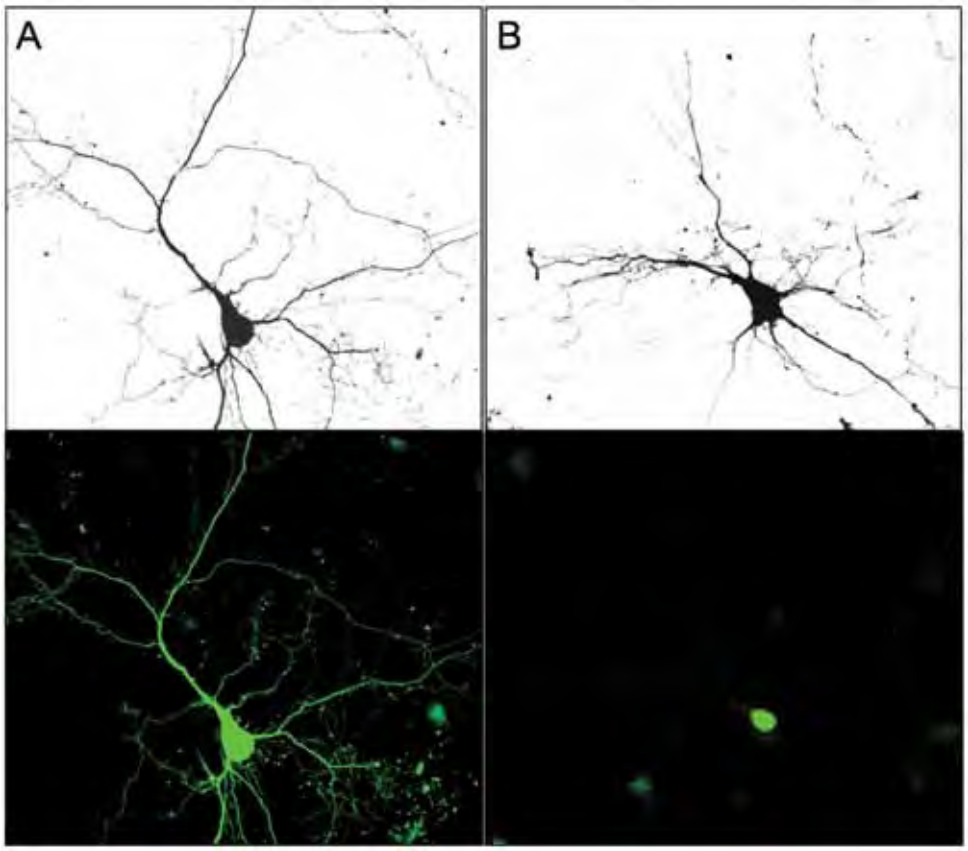

Figure 3. GFP-fusion proteins of two selected RBPs identified in the screen were expressed in hippocampal neurons. Whereas the RBP in panel ***A*** is expressed ubiquitously within the neuron, the RBP shown in ***B*** is restricted to the neuronal nucleus.

at the synapse. Therefore, we will focus on this set of candidates in future studies investigating the underlying mechanism and biological significance of neuronal function.

As a first step in our experimental efforts, we are interested in finding out whether the candidate RBPs directly associate with RISC. To this end, we will overexpress the RISC core protein Ago2 in Hek293 cells, together with the GFP-tagged RBPs, and perform co-immunoprecipitation experiments to reveal possible interactions. In addition, we will study a direct interaction of the RBPs with either the miRNA and/or the target transcript by performing pull-down experiments.

On a functional level, we will assess the importance of the candidate proteins for neuron maturation and dendritic spine plasticity. In the Luciferase experiments, knockdown of the candidate RBPs comparably impaired miR-134 and miR-138 function. Both miRNAs are known to negatively regulate the size of dendritic spines and, in the case of miR-134, promote dendritic outgrowth (Schratt et al., 2006; Fiore et al., 2009; Siegel et al., 2009). Since the selected candidates have been shown to localize to dendrites and synaptic sites, one can surmise that they regulate miR-134 and miR-138 function in the context of spine maturation and dendritic branching. Thus, we plan to knock down the RBPs in neurons under conditions of elevated miR-134/miR-138 activity to see whether we can counteract the neuronal phenotypes of miR-134/miR-138 overexpression.

Conclusions

We believe that it is feasible to perform a large-scale RNAi screening experiment in primary mouse neurons. We have already identified a small number of RBPs necessary for miRNA-mediated translational repression in neurons that so far had not been associated with miRNA function in other cell types. Thus, it is likely that neuron-specific miRNA regulators exist. They could prove to be critically involved in the modulation of miRNA-regulated local protein synthesis at synaptic sites and might present a new way to couple changes in neuronal activity to the regulation of protein levels at the synapse.

Acknowledgments

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Visualizing mRNA Trafficking and Local Translation Within Individual Neurons

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Introduction

Neurons are remarkably polarized cells. They elaborate processes that extend great distances from the cell soma in order to form distinct subcellular compartments such as growth cones and synapses. Stimulus-induced changes in the structure and function of these compartments are essential to the formation and plasticity of neural circuits (Kandel, 2001). The persistence of such changes requires new transcription and translation, raising the question of how gene expression can be spatially restricted within neurons. Studies in many systems have indicated that mRNA localization and regulated translation provide one solution (Martin and Ephrussi, 2009). Translation of localized transcripts within distinct subcellular compartments in neurons has been shown to occur at several time points: in growth cones during axon guidance and synapse formation (Lin and Holt, 2008), at synapses during synaptic plasticity (Wang et al., 2010), and in axons during injury-induced regeneration (Willis and Twiss, 2006).

A challenge to the study of local translation in neurons is the need to study mRNA localization and protein synthesis within discrete compartments, which cannot always be easily separated from the rest of the cell. This challenge has necessitated the development of novel tools and approaches for isolating pure, or highly enriched, subcellular compartments and using these:

- To identify localized transcripts;
- To characterize and elucidate the mechanisms underlying their localization; and
- To characterize and elucidate the mechanisms whereby stimulation regulates their local translation.

In this chapter, we will describe techniques for identifying localized mRNAs and for visualizing their localization and translation within neurons.

Methods for Identifying Localized Transcripts Within Neurons

Determining the population of mRNAs present in axons or dendrites requires a means of isolating each of these compartments from the cell body. The purity of the neuronal-process preparation is critical because the amount of RNA that is present in processes is orders of magnitude smaller than the amount of RNA present in neuronal or glial cell bodies, such that even minimal somatic contamination can overwhelm detection of process-localized mRNAs. Investigators have devised a number of strategies to address this problem:

- Biochemical fractionation of CNS to enrich for synaptic terminals;
- Dissection of dendritic domains within brain tissue; and
- A variety of methods to mechanically separate neuronal processes from somata of cultured neurons.

J. Eberwine and colleagues pioneered a method in which individual dendrites of dissociated neurons in culture are transected and then aspirated into a micropipette containing buffer and reagents for T7-RNA amplification (Miyashiro et al., 1994). This method has been used to generate probes for microarray analysis and has led to the identification of approximately 400 dendritically localized transcripts (Eberwine et al., 2001a). T. Suzuki and colleagues (Tian et al., 1999) isolated RNA from rat forebrain postsynaptic density (PSD) fractions and used this approach to identify 130 transcripts present in this synaptic fraction. L. Bloch and colleagues (Zhong et al., 2006) dissected the stratum radiatum from rat hippocampal slices, purified its RNA, and used this sample as starting material to identify 154 "localized" transcripts by microarray analysis.

Our lab, in turn, has developed methods for culturing rodent hippocampal and cortical neurons on Millipore filters (Millipore, Billerica, MA) containing 1-μm or 3-μm pores through which neuronal and glial processes, but not cell bodies, can penetrate (Poon et al., 2006). Harvesting the bottom, process surface with a cell scraper provides a means of mechanically separating somata from axons and dendrites. The composition and separation of the two surfaces can be assessed by confocal optical sectioning of cultures stained as follows: using DAPI to detect nuclei, antibodies to NeuN to mark neuronal nuclei, GFAP to mark astrocytes and astrocytic processes, MAP2 to label dendrites, and tau to label axons. This technique revealed that with a 1-μm or 3-μm pore, the bottom surface was devoid of cell bodies and contained exclusively axons, dendrites, and glial processes; it further revealed that with 3-μm pores, 19% of dendrites penetrated to grow along the bottom surface.

To measure the purity of the soma/process RNA preparation, we performed quantitative (qPCR) for somatically restricted transcripts (e.g., the small nuclear ribonucleoprotein SM51) and for dendritically localized mRNAs (e.g., αCaMKII and MAP2). These analyses revealed that the process prep was enriched for known process-localized mRNAs and contained minimal somatically restricted transcripts. Microarray analysis of RNA purified from process and somatic/whole-cell compartments led to the identification of approximately 100 process-localized

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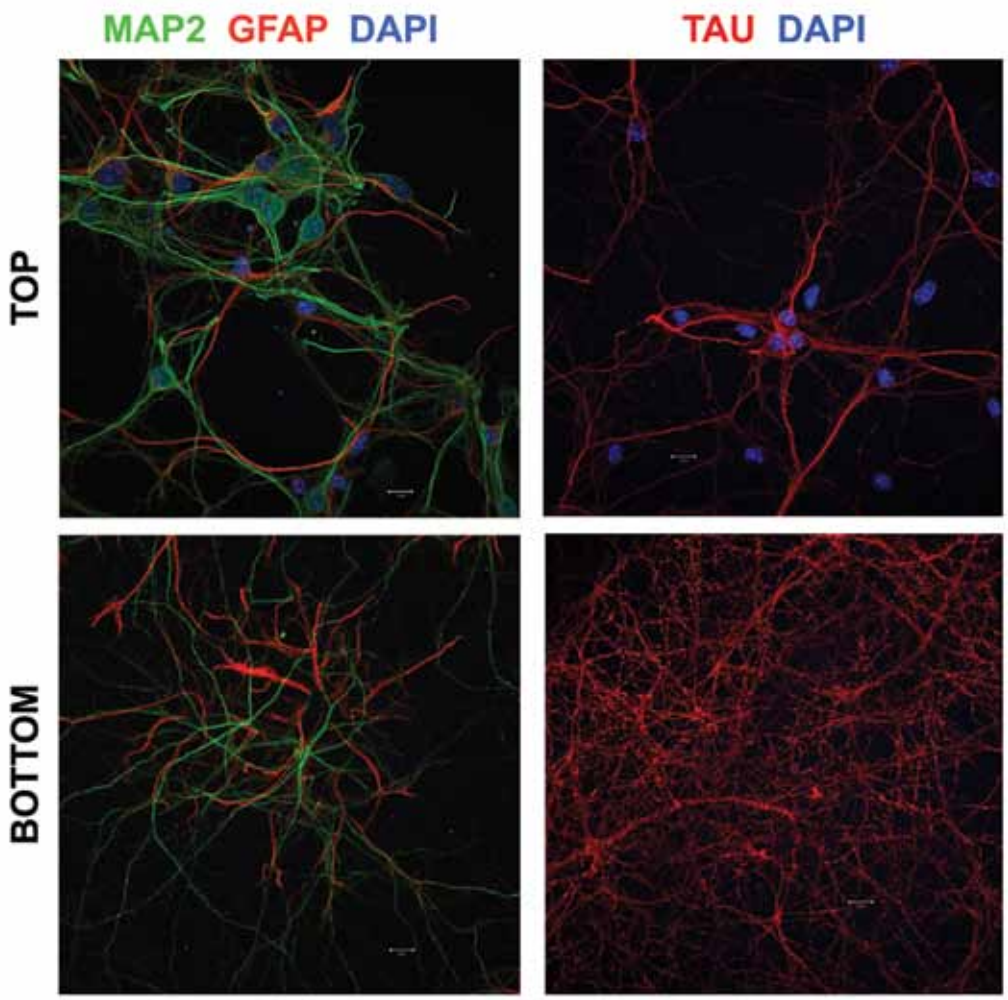

Figure 1. Rat hippocampal neurons were cultured on Millipore filters etched with 1-μm pores. Following fixation at 21 DIV, they were processed for immunocytochemistry with anti-MAP2, anti-tau, and anti-GFAP antibodies and counterstained with DAPI to visualize dendrites, axons, glial processes, and nuclei, respectively. Confocal sections of the top and bottom (separated by a 10-μm-thick filter) revealed that dendrites, axons, and glial processes grew through the pores and along the bottom surface but that cell bodies were confined to the top surface.

mRNAs; fluorescent *in situ* hybridization (FISH) of 19 of the identified localized transcripts revealed that all were present in dendrites. Similar methods have been used to isolate axonally localized mRNAs from dorsal root ganglion (DRG) neurons (Zheng et al., 2001) and sensory neurons (Cox et al., 2008).

Another method for mechanically separating neuronal processes from cell bodies (pioneered by N. Jeon and colleagues) involves growing neurons in compartmentalized microfluidic chambers (Park et al., 2006). In this method, neurons are plated into platforms in which processes and somata are separated by a physical partition containing embedded micrometer-sized grooves along which axons and dendrites, but not cell bodies, can traverse. By varying the distance of the partitions, one can obtain preparations containing pure axons, which extend greater distances than dendrites. This approach has been used by Taylor and colleagues to identify axonally localized transcripts in mature rodent cortical neurons (Taylor et al., 2009)

Neurites can be easily separated from the cell bodies of invertebrate neurons; in fact, neurites will remain viable in culture and continue to grow even in the absence of a cell body. We have used sharp electrodes to sever neuronal processes from *Aplysia* sensory

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neurons, isolating RNA from the pure neurites and using this sample as the starting material for cDNA library synthesis (Moccia et al., 2003). The sequencing of clones from this library revealed approximately 250 localized mRNAs. As described below, the ability to obtain viable preparations of isolated processes is especially advantageous for studies of local translation at synapses.

Surprisingly little overlap is revealed by comparing the lists of dendritically localized mRNAs in rodent hippocampal neurons, identified using distinct approaches (Tian et al., 1999; Eberwine et al., 2001b; Poon et al., 2006; Zhong et al., 2006). The significance of these findings is not clear: They may indicate that a potentially very large population of mRNAs localizes to dendrites and/or that the population changes with development and with neuronal activity. Critically, however, they underscore the necessity of confirming localization by *in situ* hybridization (ISH), as described in the following section.

Detecting mRNA Localization Using *in situ* Hybridization

The methods described above for unbiased identification of localized transcripts within neurons are prone to problems with specificity. The major factor that contributes to the identification of false-positives is somatic contamination in the starting material. As a consequence, it becomes critical to confirm the localization of any identified transcripts using ISH.

Localization by ISH can be determined in dissociated cultured neurons and/or in tissue sections. The advantage of assessing localization in dissociated cultured neurons is that it is easier to resolve individual axons and dendrites than in tissue sections, where dense packing limits one's ability to discern individual axonal and dendrite processes. The major advantage of tissue sections is that they represent a more physiological state and feature intact brain architecture. Performing ISH in both preparations is ideal, since one can assign localization to a specific compartment more easily in dissociated cultures, and one can confirm that this localization occurs in the intact developing or adult brain in tissue sections.

Refining techniques for *in situ* hybridization

A consequence of the low amount of RNA in processes relative to cell bodies is that the signal in the cell bodies is often saturated before signal in the processes can be detected. In many published studies and publicly accessible databases, such as the Paul Allen Brain Atlas, the exposure time for ISH is stopped as soon as signal is observed in the cell body, limiting the usefulness of these resources for identifying localized transcripts. The requirement for long exposure times also underscores the importance of including negative controls in ISH studies. In addition to sense controls, ISH for transcripts that are known to be somatically restricted is essential. Additionally, the use of more than one riboprobe, covering distinct sequences in the RNA but showing the same pattern of localization, serves as a useful control. Finally, FISH is compatible with immunocytochemistry using some antibodies, such as anti-MAP2 antibodies, which allows for the colocalization of RNA signal with MAP2 immunoreactive dendrites (Poon et al., 2006).

Highly sensitive methods for FISH have greatly facilitated the identification of localized transcripts in neurons and other asymmetric cells. One study by H. Krause and colleagues (Lécuyer et al., 2007) used high-throughput FISH analyses to show that more than 70% of mRNAs showed very specific, hitherto unappreciated patterns of subcellular localization in *Drosophila* embryos. We have used FISH with digoxigenin or biotin-labeled riboprobes, from approximately 300 to 600 bp in length, coupled with tyramide-signal amplification, to detect localization of mRNAs in a variety of prepared samples: cultured hippocampal neurons, hippocampal sections, cultured *Aplysia* sensory-motor neurons, cryostat sections of *Aplysia* ganglia, and whole-mount *Aplysia* CNS (Lyles et al., 2006; Poon et al., 2006; Wang et al., 2009). In each of these preparations, we were able to optimize conditions in order to detect specific localization in distal neuronal processes with antisense (but not sense) probes. Important variables included the following: hybridization temperature, stringency of washes, and time of the tyramide signal amplification.

R. Singer and colleagues have developed methods for highly quantitative FISH by synthesizing several oligonucleotide probes, targeting adjacent sequences on an mRNA, and conjugating fluorochromes to specific sites on each oligonucleotide. This technique has made it possible to calibrate the signal to a known concentration of labeled oligonucleotide probes and, with appropriate imaging, to quantify the number of RNA molecules being detected (Femino et al., 2003). Although this method is more expensive than FISH using *in vitro* transcribed riboprobes, it is

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advantageous because it is quantitative and sensitive. Further, combining the labeling of oligonucleotides with a variety of distinct fluorophores allows investigators to detect multiple transcripts within a single sample.

The absence of an ISH signal does not necessarily mean that the transcript is not localized. Thus, transcripts may be present at low abundance (below the threshold for detection) or present in structures (e.g., RNA transport granules) that are inaccessible to hybridization by the riboprobe or oligonucleotides. Many ISH protocols include a protease step to digest proteins binding the RNA and thereby increase transcript accessibility during the hybridization step. As increasingly sensitive FISH protocols are developed and utilized to analyze mRNA localization in neurons, it is likely that highly distinct patterns of subcellular mRNA localization will emerge, analogous to what has been observed in *Drosophila* embryos (Lécuyer et al., 2007). Identifying these specific sites of localization may reveal subcellular compartments that had previously been unappreciated; thus, these findings may lead to a more detailed and nuanced understanding of neuronal architecture.

Dynamic Imaging of mRNA Localization in Neurons

ISH analysis provides only a snapshot of mRNA localization within neurons, and thus cannot provide much insight into mechanisms whereby the transcript is localized. Methods for dynamic imaging of mRNA transport in living neurons are needed to gain a better understanding, for example, of whether mRNAs are actively transported to particular sites, whether their localization results from anchoring within specific compartments, or whether their localization results from differential stability within the neuron.

Methods for directly visualizing RNA localization in neurons can be divided into two categories:

1. Methods using probes that hybridize to, and track the movement of, endogenous transcripts; and
2. Methods that track the movement of heterologously expressed mRNAs within neurons.

The advantage of the first approach is that the mRNAs are expressed at endogenous levels and undergo native processing within the nucleus and cytoplasm. It is nonetheless difficult to design probes that will hybridize and brightly label endogenous transcripts, and the possibility exists that any hybridizing reagent will interfere with the ability of the endogenous RNA to interact with its normal protein and RNA partners. The advantage of the second approach is that one can generate transcripts that are brightly labeled to facilitate live cell imaging. The disadvantages include saturation of trafficking mechanisms as a result of high levels of overexpression, as well as artifacts resulting from the fact that exogenously expressed cDNAs do not undergo the same processing in the nucleus as genomically transcribed RNAs. Studies of *oskar* mRNA localization in *Drosophila* have clearly illustrated that the "nuclear history" of the mRNA is critical to its subsequent localization within the cytoplasm (Hachet and Ephrussi, 2004).

Molecular beacons

One method for visualizing endogenous mRNAs is through the use of molecular beacons (Santangelo et al., 2006). Molecular beacons are small hairpin-loop nucleic acids with a fluorochrome attached to one of the free ends and a molecule that quenches the emission of that fluorophore attached to the other end. When the beacon hybridizes to its target mRNA sequence, the hairpin-loop structure is linearized such that there is no longer any quenching of the fluorochrome. In this way, the beacon-bound target is detectable by live-cell fluorescence microscopy. The beacon must be able to hybridize to its target and be stable within the cell in order to be effective. An improvement on this method involves the use of molecular beacons directed against adjacent sequences in the target mRNA and containing fluorophores at the free ends that, when hybridized to the target, will lead to fluorescent resonance energy transfer (FRET). This enhanced technique has been used to follow the trafficking of viral RNAs in cells (Santangelo et al., 2006). One limitation of the molecular beacon approach is that the signal is not very bright, since usually only one fluorochrome is present.

Microinjecting *in vitro*–transcribed, fluorescently labeled transcripts

Another method for visualizing transport of exogenously introduced mRNAs is by microinjection of *in vitro*–transcribed, fluorescently labeled transcripts in cells. Labeled transcripts can be generated by including nucleotides coupled to fluorophores (e.g., with cyanine dyes or Alexa fluors) in the *in vitro* transcription reaction. The fluorescently labeled mRNAs can then be microinjected into neurons, preferably into the nucleus where they can potentially bind appropriate RNA-binding proteins necessary for their cytoplasmic localization, and be tracked by live-cell microscopy. This approach has been used to track RNAs moving in granules along microtubules (Tübing et al., 2010), and, in

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conjunction with mutational analyses, to identify *cis*-acting elements required for RNA localization (Bullock and Ish-Horowicz, 2001; Wilkie and Davis, 2001).

MS2 and lambda N systems

Whereas molecular beacons and *in vitro*–transcribed RNAs must be introduced into cells by microinjection or by cell-penetrating peptides, the MS2 (Bertrand et al., 1998) and lambda N (Daigle and Ellenberg, 2007) systems provide a means of genetically encoding fluorescent markers of specific mRNAs in cells and animals. Both approaches contain two components, which we will describe for lambda N. The first component encodes the RNA of interest, into which a series of 15-hairpin-loop RNA elements, called box B elements, are engineered into the 3′ untranslated region (UTR). It is useful to have this construct encode a fluorescent protein in order to visualize the efficient expression in transduced, transfected, or microinjected cells. The second component encodes the 22-amino-acid-long bacteriophage protein lambda N, fused to a nuclear localization signal (NLS) and to three copies of enhanced green fluorescent protein (eGFP, or any other fluorescent protein). When the second protein is expressed in a cell, the NLS causes it to accumulate within the nucleus. When, however, it binds to the target RNA containing the box B binding sites, the high binding affinity overrides the nuclear localization, and one can follow the labeled mRNA as it is transported into the cytoplasm. The lambda N and MS2 approaches have gained widespread use for tracking RNA movement in a number of cell types, including neurons.

Several variables must be optimized for the system to work in a particular experimental preparation. We have found, for example, that a single NLS is not very efficient for nuclear import in primary neurons because it leads to background signal in the cytoplasm even in the absence of box B–containing RNA. However, three copies of the NLS decrease cytoplasmic background. In addition, one can test the efficiency of distinct NLSs, for example, the M9 NLS or the SV40 large T-antigen NLS. Another variable to optimize is the number of fluorescent proteins fused to the lambda N or MS2 protein, with the aim of achieving a signal that is bright enough to detect and follow over time but that does not add excessive molecular weight to the localized transcript. It may also be necessary to change the location of the box B sites in the 3′UTR. Finally, one can experiment with increasing the number of box B sites in the target RNA in order to increase the signal, again, without imposing an excessively large molecular weight on the transported RNA.

Methods for Visualizing Local Translation in Living Cells

Novel methods have been developed to determine whether a specific mRNA is locally translated in neurons. Here we will focus on translational reporters encoding photoconvertible fluorescent proteins, which we have used to visualize translation at *Aplysia* sensorimotor synapses during long-term neuronal plasticity (Wang et al., 2009). Our aim here is to discuss some of the critical variables that apply when performing and interpreting this type of experiment.

Whether or not to include the entire RNA transcript

When designing translational reporters for local translation, one must first determine which regions of the RNA are required for mRNA localization and regulated translation. One possibility is to include the entire transcript, fusing a photoconvertible fluorescent protein (the reporter) to the coding region. Another possibility is to fuse the 5′ and 3′UTRs of the localized mRNA to the reporter. The rationale for the latter is that many studies have indicated that localization elements are often (but not always) contained within the 3′UTR, whereas translational regulation is often encoded, at least in part, by the 5′UTR. To confirm that a reporter accurately reflects the localization of the endogenous RNA, one can perform double-label FISH for the endogenous and reporter RNAs. It is worth noting that this approach can be used in conjunction with mutational analyses in order to determine the specific RNA elements involved in RNA localization.

Detecting new translation using photoconvertible fluorescent proteins

Having a translational reporter that encodes a photoconvertible fluorescent protein provides a means of monitoring new, local translation because one can photoconvert all preexisting protein from green to red and then monitor new translation as the appearance of new green signal. A critical concern in these experiments is to verify that the newly synthesized (green) protein is synthesized locally, as opposed to being made in the soma, and then being transported to a distal locale. This can be challenging because the protein takes some time to fold and mature before it actually emits fluorescence, and during this time it can diffuse or be transported within the cell. Further, since there is always more translation in the soma than in neuronal processes,

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the signal from somatically synthesized protein can saturate local translation.

The time it takes for a fluorescent protein to fold and fluoresce within a cell is not easy to measure. In our hands, in *Aplysia* neurons at room temperature, we can detect the appearance of newly synthesized (green) reporter within approximately 10 min. One way to limit the diffusion of protein from its site of synthesis during this time is to attach a signal for membrane anchoring, e.g., for myristoylation or palmitoylation (Aakalu et al., 2001). This approach has the advantage of concentrating the signal and making it more easily detectable. Of note, however, we have found that, unlike soluble proteins, myristoylated reporter proteins in *Aplysia* neurons do not colocalize with the reporter RNA and in fact are often separated by tens of microns; this characteristic suggests that the protein may be transported following synthesis to distinct membrane domains.

In *Aplysia*, the problems with somatically synthesized proteins diffusing can be directly addressed by simply severing and removing the cell body, photoconverting the remaining neurites, and visualizing new (green) protein synthesis, which can have occurred only locally. This is a major advantage of this system over vertebrate neurons, where neuronal processes deprived of their cell bodies do not survive in culture.

Controls in these types of experiments include inhibition of protein synthesis with translational inhibitors such as anisomycin, cycloheximide, or emetine to ensure that any newly detected green protein results from local translation. It is also important to include volume-filling controls to ensure that any increases in signal do not result simply from changes in the local volume of a structure; we have found that the photoconverted, soluble red dendra2 protein serves this purpose well.

An increasing number of photoconvertible fluorescent proteins can be used for these experiments. Each has slightly distinct characteristics (Huang et al., 2009; McKinney et al., 2009). Some, such as Kaede, are tetrameric; others, such as dendra2 and mEos, are monomeric. Dendra2 is advantageous for certain experiments because a high-affinity anti-dendra2 antibody is available; mEos2 is advantageous because it is more photostable than dendra2, and because, unlike its precursor mEos, it photoconverts efficiently at 37°C. As new photoconvertible fluorescent proteins are being generated, it is worth investigating these before generating constructs for imaging, taking into consideration several features: whether they are monomeric or multimeric, how bright and photostable they are, what wavelengths they emit, how much light is required to photoconvert them, how stable the photoconverted product is, and whether or not there are any antibodies available to detect the expressed fusion protein.

Conclusion

mRNA localization and regulated translation have been found to play critical roles in a wide range of physiological processes in the brain. The spatial restriction of gene expression that mRNA localization affords allows individual synapses and processes to autonomously regulate their protein composition in response to distinct stimuli, greatly expanding the computational capacity of neural circuits. However, this spatial restriction of gene expression also poses technical challenges to the study of mRNA localization and local translation because one cannot simply analyze mRNAs and proteins in whole-cell or tissue homogenates. Rather, genomic-scale approaches to identifying the population of localized transcripts will require new techniques for purifying subcellular neuronal compartments. Similarly, understanding how mRNAs localize and how their translation is regulated within specific compartments will require the development of single-cell-level imaging techniques for visualizing each process within neuronal subcellular compartments (Weil et al., 2010).

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Posttranscriptional Regulation of Intrinsic Plasticity

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Introduction

In 1894, Santiago Ramon y Cajal proposed that learning results from changes in synaptic strength (Cajal, 1894). This idea is consistent with the basic premise for memory formation and suggests that modifying independent synapses provides a large capacity to store information (Martin et al., 2000). Nearly 80 years after Cajal's initial proposition, a landmark study by Bliss and Lomo demonstrated that excitatory synapses in the hippocampus can undergo persistent changes in synaptic strength that can be sustained for hours or even several days. These persistent changes are the cellular basis for a model of learning and memory that is commonly referred to as "long-term potentiation" (LTP) (Bliss and Lomo, 1973).

Today, we know that synaptic strengthening requires changes in the number and/or conductance of glutamate receptors (specifically AMPA and NMDA type) with the induction of LTP. (Malenka and Bear, 2004; Shepherd and Huganir, 2007; Kessels and Malinow, 2009). However, new questions emerge if we consider the fact that LTP is Hebbian in nature, i.e., that synapses can be modified by previous experience (Hebb, 1949). How do changes in glutamate receptor activity influence the propensity of a synapse and nearby synapses to respond to presynaptic release? What are the underlying mechanisms that change postsynaptic responsiveness or, in other words, set the plasticity threshold, a process that Abraham and Bear termed "metaplasticity" (Abraham and Bear, 1996)? Can these metaplastic changes be compartmentalized in such a way that they are restricted to a synapse, a dendritic branch, or a dendritic tree?

Anatomy of a Neuron

The nature of a neuron is polarized. It naturally divides into microdomains of specifically targeted proteins that facilitate the structural and functional differences between the dendrites and the axon. Dendrites themselves can be subdivided into individual compartments based on several factors: their proximity to the soma, their branching patterns from the apical trunk, and the synaptic inputs they receive (Spruston, 2008). The proteins that make up these specialized microdomains determine the synaptic efficacy of the individual compartment.

In neurons, the relative density and dendritic localization of ion channels play important functional roles in synaptic integration, plasticity, and neuronal excitability (Frick and Johnston, 2005). Historically, dendrites were viewed as electrically passive, in contrast to the electrical excitability of the soma (Johnston et al., 1996). However, this idea has been found to be inconsistent with the observation that action potential firing exceeds what would be predicted by the summation of LTP-induced EPSPs. Bliss and colleagues described this phenomena as a "nonsynaptic component of LTP" (Bliss and Gardner-Medwin, 1973). We now know that neuronal dendrites are not passive and that, in fact, they have a rich and complex distribution of ion channels (Zhang and Linden, 2003; Frick and Johnston, 2005; Bloodgood and Sabatini, 2008).

The first indication that voltage-gated ion channels are "plastic" during LTP came from a series of experiments that initially discovered that sodium-dependent action potentials can propagate back into the dendrites from the soma (i.e., back-propagating action potential, or bAP) (Johnston et al., 1996). To test whether LTP causes changes in ion channel properties that alter membrane excitability (often referred to as "intrinsic plasticity"), bAPs were paired with synaptic stimulation (Frick et al., 2004; Frick and Johnston, 2005). Using calcium imaging and dendritic recordings, Johnston and colleagues found that, at the site of stimulation, there was an increase in bAP amplitude and an enhanced calcium signal, suggesting an increase in local dendritic depolarization (Frick et al., 2004). To account for this unexpected heightened excitability, changes in channel-gating and endocytosis of potassium channels have been proposed (Frick et al., 2004; Kim et al., 2007). Another possible mechanism, as our work suggests, is that NMDA activity triggers the posttranscriptional repression of potassium channel mRNA translation (Raab-Graham et al., 2006).

These critical experiments open the field to questioning how changes in synaptic efficacy are coupled to changes in dendritic excitability, both locally and globally (Zhang and Linden, 2003; Frick and Johnston, 2005; Kim and Linden, 2007). Thus, this chapter will focus first on the importance of local translation and repression during LTP. Second, it will discuss the identification and the regulation of local translation of ion channel mRNAs in neuronal dendrites. Third, it will describe how site-specific changes in dendritic excitability are important for synaptic plasticity and how, if left unchecked, they may be involved in neurodegeneration.

Significance of mRNA Translation and Repression in Long-term Potentiation

LTP consists of two phases: early and late. It is widely believed that late-phase LTP serves as a useful model

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for the consolidation phase of memory formation (Pittenger and Kandel, 2003). For several years, we have known that the conversion of early LTP to late phase requires protein synthesis (Krug et al., 1984; Frey et al., 1993; Abel et al., 1997). In 1983, Bliss and colleagues set out to examine changes in protein synthesis during late LTP using two-dimensional protein electrophoresis of ^{35}S-methionine–labeled proteins that were synthesized during LTP *in vivo* (Fazeli et al., 1993). Remarkably, this technique revealed not only an increase in protein synthesis but also a corresponding decrease in the relative abundance of certain proteins. Interestingly, in their discussion, the authors stated that LTP was accompanied by the "reduction in synthesis (or an increase in degradation)." Since then, proteosome-mediated protein degradation during neuronal activity has been an active area of research (Hegde and DiAntonio, 2002). Nonetheless, the idea of suppression of protein synthesis has been largely ignored.

mTOR, Plasticity, and Memory

Consistent with the requirement for protein synthesis during late-phase LTP, signaling of the mammalian target of rapamycin (mTOR) pathway is required for the maintenance of late LTP. mTOR is a serine/threonine kinase whose primary function is to promote mRNA translation initiation (Hay and Sonenberg, 2004). In hippocampal slices, elegant electrophysiological experiments that block mTOR signaling via the mTOR-specific inhibitor rapamycin, prior to LTP induction, reduced the magnitude of LTP for more than 5 h. (Tang et al., 2002; Cammalleri et al., 2003; Vickers et al., 2005). Further, inventive genetic approaches that resulted in increased basal phosphorylation of mTOR in mice have shown enhanced late LTP (Hoeffer et al., 2008). In line with the findings of these reports, behavioral tasks designed to assess learning and memory in rodents have indicated that mTOR activity is required for memory formation, consolidation, and reconsolidation (Casadio et al., 1999; Parsons et al., 2006; Bekinschtein et al., 2007; Blundell et al., 2008; Hoeffer et al., 2008).

Local Translation and Neuronal Plasticity

Unexpectedly, mTOR has been found both in the cell body and in the dendrites of neurons, suggesting that protein translation also occurs in the dendrites (Tang et al., 2002). These data challenge the view that all proteins are synthesized in the cell body and transported to the dendrites. Further support for the dendritic protein translation hypothesis has been found in the ability to detect polyribosomes, mRNAs, translation machinery, as well as components of the secretory pathway (endoplasmic reticulum and golgi membranes) in dendrites (Bramham and Wells, 2007). Moreover, numerous biochemical assays on synaptosomes (isolated presynaptic and postsynaptic nerve endings) and severed dendrites have demonstrated the incorporation of radioactive amino acids in proteins in the absence of cell bodies (Schuman, 1997; Steward, 1997). Finally, the use of molecular tools that we and others have developed to visualize local protein synthesis in dendrites have advanced our understanding as to how local translation contributes to neuronal plasticity (Aakalu et al., 2001; Raab-Graham et al., 2006). Thus, local translation provides the unique advantage over somatic translation and protein trafficking by making available a specific source of new proteins in response to site-specific changes in synaptic strength (Schuman et al., 2006).

Characterization of Dendritic Kv1.1 Local Synthesis

Based on the evidence that voltage-gated ion channels are important in dendritic signaling, Patrick Haddick and I performed our own screen, looking for synaptic mRNAs while I was a postdoctoral fellow in the laboratory of Dr. Lily Jan at the University of California, San Francisco. Using microarrays and quantitative real-time PCR, we compared mRNA isolated from synaptosomes with mRNA isolated from the hippocampus. We determined that 4% of all transcripts assayed, including 202 known genes, reside at the synapse. These mRNAs report a synaptosome "intensity value" greater than αCaMKII (a message that has been previously reported to be localized to dendrites) and a synaptosome-to-hippocampus ratio of greater than 1.2. Interestingly, several transcripts encoding ion channels and neurotransmitter receptors known to be involved in synaptic plasticity were enriched in the synaptosomal fraction.

The first transcript we characterized from our microarray data was the dendrotoxin-sensitive, voltage-gated potassium channel Kv1.1, which controls the frequency of the action potential (Tanouye and Ferrus, 1985). Although a dendrotoxin-sensitive Kv current has been described in CA1 pyramidal neurons, the molecular identity of this current is unknown (Chen and Johnson, 2010) and the specific role for Kv1.1 in the dendrites of hippocampal neurons has not been established.

We verified our microarray data by detecting endogenous Kv1.1 mRNA in dendrites of cultured

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hippocampal neurons using *in situ* hybridization. The results were surprising because the prior thinking held that Kv1.1 was expressed exclusively in the axon in the hippocampus (Schechter, 1997; Southan and Owen, 1997; Geiger and Jonas, 2000; Monaghan et al., 2001; Raab-Graham et al., 2006). Thus, Kv1.1 mRNA trafficking provides a mechanism for the protein to escape the restrictive protein-encoded trafficking signals that direct the protein to the axon in order to be expressed in the dendrites in an activity-dependent manner.

To confirm the dendritic localization of the channel, we demonstrated that the mTOR inhibitor rapamycin increased the total Kv1.1 protein in the CA1 region of the hippocampus and the surface expression in cultured neurons (Fig. 1*A*,*B*). These results were contrary to what we expected, because mTOR activity was supposed to promote translation. However, what we found was that mTOR activity represses the translation of Kv1.1 mRNA.

To verify this unusual finding, we decided to visualize local dendritic protein synthesis of Kv1.1. To do this, we developed an improved method for visualizing local translation in dendrites. One technical challenge in the field was separating existing proteins from newly synthesized proteins. We were able to solve this problem through the use of the photoconvertible protein Kaede (Mizuno et al., 2003) and multiphoton microscopy. Ultraviolet (UV) light induces a specific proteolytic cleavage of Kaede, converting its fluorescence from green into red. This property allowed us to develop a translational reporter by fusing Kaede to Kv1.1. Newly synthesized Kaede–Kv1.1 is distinguished

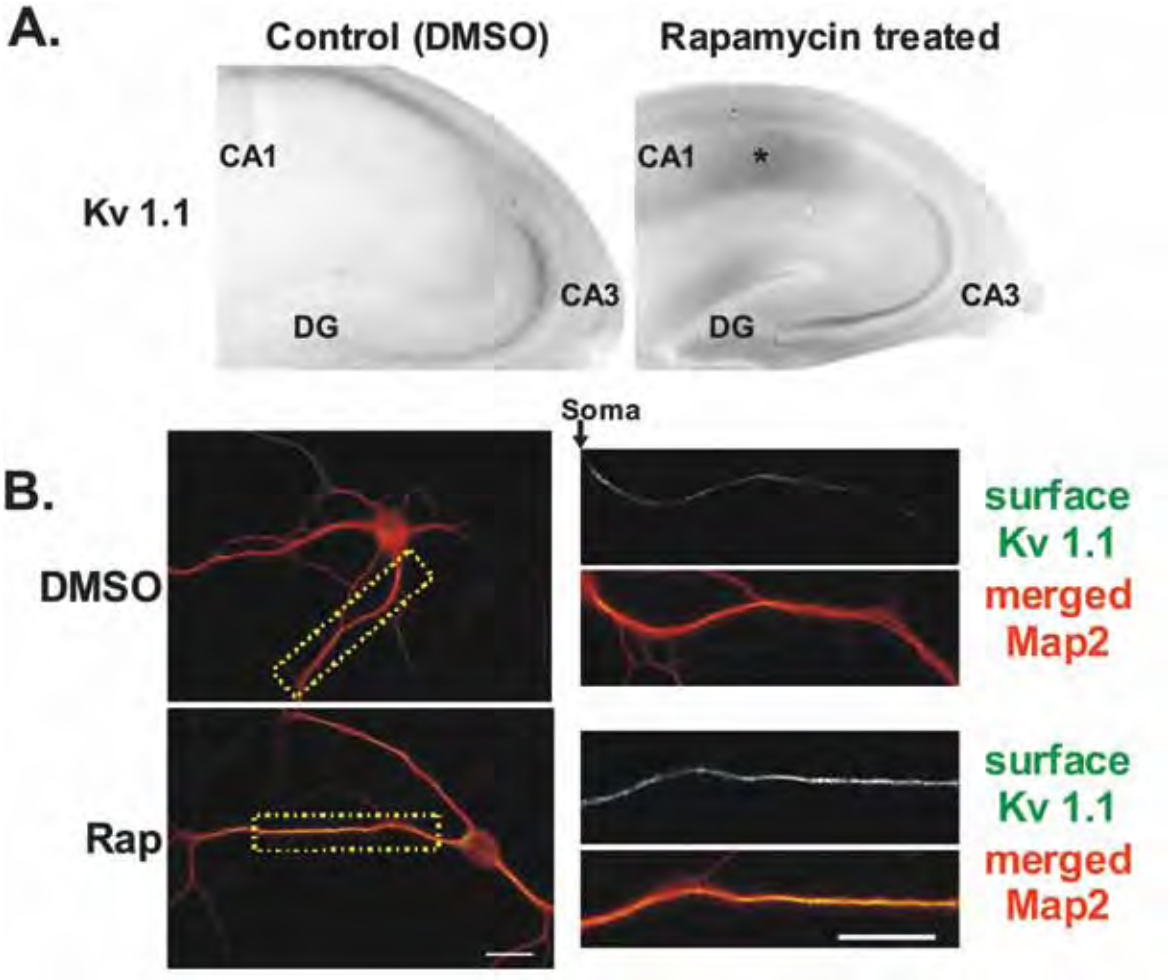

Figure 1. Inhibition of mTOR by rapamycin increases the local translation of Kv1.1 mRNA in central neuronal dendrites. ***A***, Acute hippocampal slices treated with carrier or rapamycin (200 nM) for 75 min. Slices were fixed and stained with an antibody against Kv1.1. Note the significant increase in Kv1.1 expression in CA1 and dentate gyrus with mTOR inhibition by rapamycin. ***B***, Surface staining of Kv1.1 protein of treated hippocampal neurons. Map2-positive dendrites show a significant increase in Kv1.1 protein in the dendrite greater than 50 μm from the soma. Scale bar, 20 μm. Raab-Graham et al. (2006), their Fig. 1*A*,*D*, reprinted with permission.

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by its green chromophore and spectrally separated from the previously synthesized red protein (Raab-Graham et al., 2006) (Fig. 2*A*). With tools in hand, we went on to demonstrate that Kaede–Kv1.1 is locally synthesized in dendrites upon inhibition of mTOR (Fig. 2*B*).

These results led us to form a model proposing that the suppression of Kv1.1 mRNA translation is an imperative positive-feedback mechanism during NMDA/mTOR–dependent neuronal activity that may be important for memory formation (Fig. 3). This model suggests that, at the site of synaptic stimulation, mTOR actively suppresses the translation of Kv1.1 mRNA, resulting in fewer channels on the surface of the dendrite, thereby creating a functional unit of the dendrite to be more excitable. Furthermore, at inactive synapses, Kv1.1 mRNA is translated and expressed on the membrane, possibly to increase the plasticity threshold.

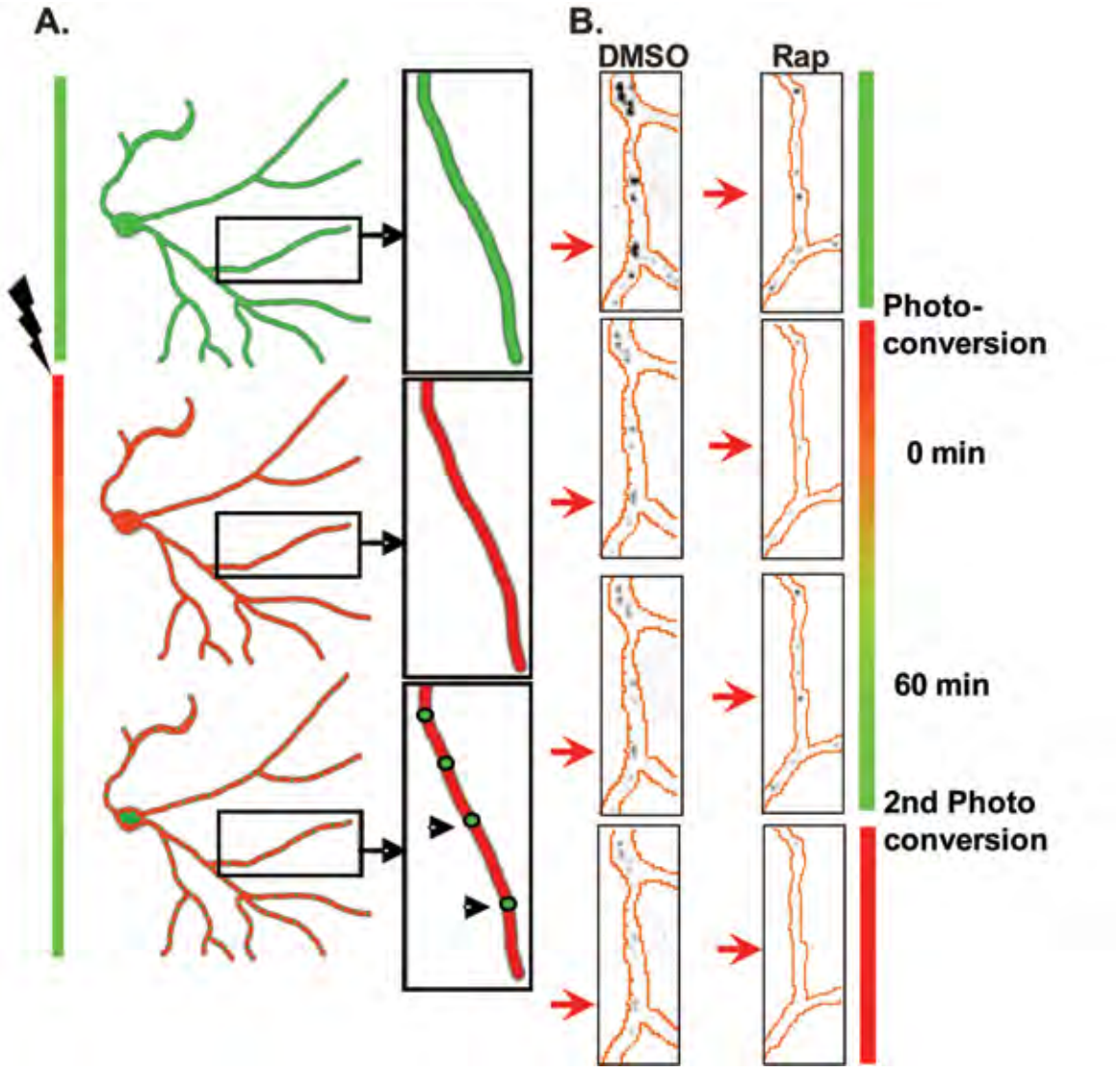

Figure 2. Local translation assay using photoconvertible protein Kaede. ***A***, Schematic of local translation assay using the photoconvertible protein Kaede. Left side shows neuron with boxed dendrites; right side shows enlargement. Green puncta indicate local translation "hot spots." Kaede initially appears green, but with UV exposure, is converted to red. New protein synthesis is monitored by the appearance of new green protein over time (arrowheads). ***B***, Live imaging of neurons expressing Kaede–Kv1.1 treated with carrier (DMSO) or rapamycin (200 nM) before, immediately after (0 min), and 60 min after the first UV exposure to photoconvert Kaede-Kv1.1 into red protein. Representative grayscale images show green fluorescence in neurons. Dendrite is outlined in orange within the black box, with arrows pointing to a single translational "hot spot" for Kaede-Kv1.1 (Raab-Graham et al., 2006).

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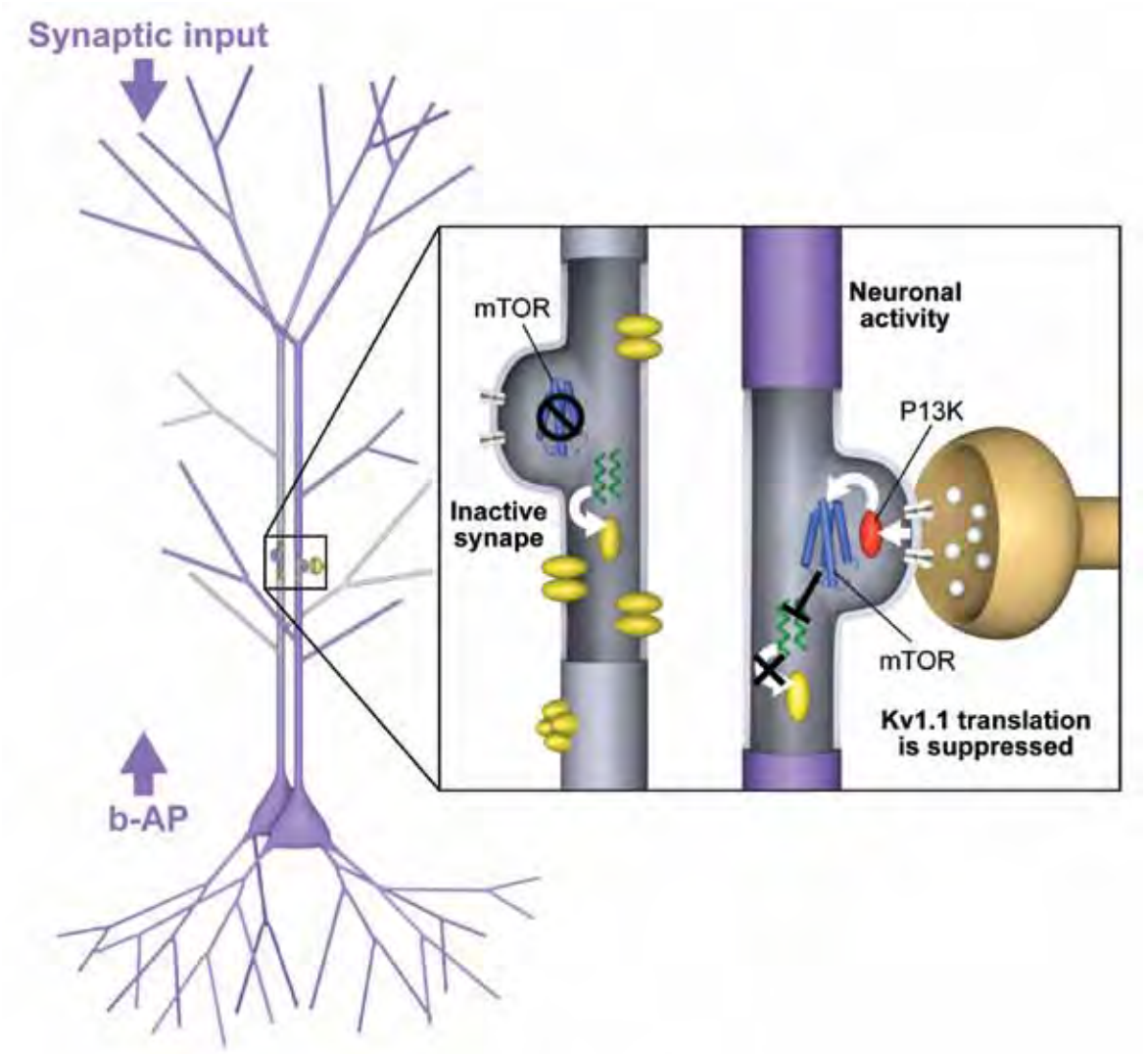

Figure 3. Model of positive feedback mechanism for the specific enhancement of dendritic excitability during NMDA/mTOR–mediated synaptic activity. Neuron on the left has an inactive synapse (gray) and mTOR is not active, thus permitting Kv1.1 local translation (green, RNA; yellow, channel subunit). Neuron on the right shows an active synapse (purple); NMDA-R (NMDA receptor) activity (white) turns on the mTOR signaling pathway via the PI3 kinase. mTOR suppresses the translation and insertion of Kv1.1 channels in dendrites. Activity-dependent changes in Kv1.1 expression may affect dendritic signaling via synaptic input and bAPs.

Misregulation of mTOR Leads to Seizure and Cognitive Decline

Patients with neurological diseases caused, at least in part, by overstimulation of the mTOR pathway experience seizures and cognitive defects. Overactive mTOR has been implicated in diseases such as epilepsy, tuberous sclerosis complex (TSC), Fragile X syndrome, and Alzheimer's disease (AD) (Meikle et al., 2007, 2008; Pei and Hugon, 2008; Zeng et al., 2009; Sharma et al., 2010), all of which have epileptic seizures associated with them. Interestingly, increased seizure susceptibility directly correlates with a decrease in *kv1.1* gene expression (Smart et al., 1998; Rho et al., 1999).

Recently, Mucke and colleagues provided evidence that the human amyloid precursor protein transgenic mouse, a model for AD, has spontaneous, nonconvulsant seizure activity in hippocampal and cortical circuits similar to what is observed

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in temporal lobe epilepsy. They suggest that the aberrant excitatory neuronal activity may lead to the cognitive impairment observed in AD (Palop et al., 2007). Both the cognitive deficits and the neuronal hyperexcitability observed in AD models parallel the phenotype observed in other mTOR-related diseases such as TSC. Furthermore, treating rat and mouse models of temporal lobe epilepsy and TSC with rapamycin eliminated the observed spontaneous seizures (Meikle et al., 2007, 2008; Zeng et al., 2009) and memory deficits observed in these rats and mice (Meikle et al., 2007; Ehninger et al., 2008). It remains to be determined whether the hyperexcitability of these neurons results from mTOR-repression of Kv1.1 mRNA translation. Reducing seizure activity in the brain may reduce the cognitive deficits observed in mTOR-related diseases such as AD and TSC. Although Kv1.1 is one of many transcripts whose translation is regulated by mTOR activity, its physiological role in controlling neuronal excitability is not compensated by other voltage-gated potassium channels (Smart et al., 1998; Rea et al., 2002; Tavazoie et al., 2005; Gong et al., 2006; Slipczuk et al., 2009).

These neurological diseases are perfect examples of how positive feedback mechanisms, if unregulated, can lead to uncontrolled neuronal excitability, thus causing neuronal circuits to become unstable and rendering the storage of new information labile (Turrigiano and Nelson, 2000). Therefore, homeostatic mechanisms need to be identified that release translational repression of Kv1.1 mRNA translation in order to prevent instability and neurodegeneration.

Considerations for Future Research

Our findings contradict the dogma that mTOR signaling promotes global cap-dependent translation. Our data suggest that cap-dependent translation of select mRNAs is induced while the translation of other mRNAs is actively repressed. In light of these data, it is interesting to reconsider the implications of earlier studies that demonstrated the reduction in late-phase LTP by the mTOR inhibitor rapamycin. It is clear that mTOR promotes the translation of important transcripts that enhance synaptic plasticity (Gong et al., 2006; Kelly et al., 2007); however, whether the mRNAs repressed by mTOR activity are memory suppressor proteins is a key unanswered question. We recently addressed this question using a mouse model for memory impairment that overexpresses the RNA-binding protein HuD and discovered that HuD promotes the translation of Kv1.1 mRNA. CA-1 pyramidal neurons from these mice have increased Kv1.1 expression, decreased firing rates, and an increased action potential threshold. Moreover, the most striking result of this study is that calcium signals provoked by bAPs in these mice are specifically reduced in oblique dendrites when compared with wild-type littermates (P. Huang, N. Sosanya, P.Y. Chang, K. Nguyen, N.I. Perrone-Bizzozero, and K.F. Raab-Graham, unpublished observations). These data suggest that translational regulation of Kv1.1 may result in both local and global changes in intrinsic excitability and may be important for memory storage.

Determining what other mRNAs are suppressed by mTOR activity, addressing how mTOR activity represses mRNA translation, and establishing whether mTOR activity regulates small noncoding regulatory RNAs, such as microRNAs, are questions for future investigation.

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Coordinated Regulation of Synaptic Plasticity by microRNAs

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Introduction

Neurons are interconnected through specialized junctions, known as synapses, that modulate all our brain functions. Structural and functional modification of these synapses (also known as synaptic plasticity) that occurs in response to neuronal activity is a critical regulator of various nervous system functions, including long-term memory formation (Malenka and Nicoll, 1999; Sutton and Schuman, 2006). Thus, a comprehensive understanding of the mechanisms of synaptic plasticity is a prerequisite for visualizing how synaptic changes can modulate the formation of long-term memory. Current understanding of synaptic plasticity centers on remodeling the cytoskeleton, neurotransmitter receptor trafficking, and activity-dependent gene transcription mechanisms. More recently, however, protein synthesis mechanisms at synapses have emerged as critical modulators of synaptic plasticity (Malenka and Nicoll, 1999; Sutton and Schuman, 2006).

In polarized neurons, mRNAs are synthesized in the cell soma and then transported far away, to dendritic spines: a specialized biochemical compartment of the neuronal dendrite. These dendritic spines are equipped with the biochemical machinery necessary for autonomous regulation of various synaptic functions. MicroRNAs (miRNAs) are among several players that have been shown to modulate synaptic protein synthesis. These small noncoding RNAs emerged recently as critical regulators of dendritic protein synthesis.

miRNAs are ~21-nucleotide-long transcripts that can fine-tune gene expression in a variety of systems, including the nervous system. miRNAs reside in RNA-induced silencing complex (RISC) and bind to the 3′ untranslated region (UTR) of mRNAs, resulting in translational suppression (Kosik, 2006; Schratt, 2009). For example, a recent study showed that synaptic activation could release miRNA-mediated translation suppression and subsequent *de novo* protein synthesis from its mRNA target, *LimK1* (Schratt et al., 2006). Although this observation and others implicate miRNA-mediated control of protein synthesis in synaptic plasticity, the mechanistic details of this regulatory control remain largely unknown (Kosik, 2006; Schratt, 2009; Vo et al., 2010).

In a recent study, we showed that activity-dependent rapid, bidirectional modulation of RISC function can regulate synaptic protein synthesis from specific mRNAs that contain miRNA binding sites. We also observed that one of the key RISC factors (MOV10, a DEAD box helicase) is degraded rapidly by the proteasome and subsequently releases RISC-mediated translational inhibition.

In order to gain insight into the spectrum of dendritic mRNAs that are modulated through RISC's degradative control, we designed a Translational Trap method to identify miRNAs that RISC targets. This method relies on the fact that removal of the key RISC protein, MOV10, by RNA interference (RNAi)–mediated knockdown would release translational suppression. This de-repression of protein synthesis would then allow RISC-regulated mRNAs to redistribute from a translationally suppressed pool to an actively translating polyribosomal fraction. Based on this hypothesis, we performed ribosomal fractionation after MOV10 RNAi and analyzed polyribosome-associated mRNAs by quantitative PCR (qPCR) using primers specific for 54 dendritically localized mRNAs (Duncan and Gilman, 1998; Poon et al., 2006; Zhong et al., 2006). Apart from the two previously known RISC-regulated mRNAs, *LimK1* and αCaMKII, our screen identified a dozen synaptically localized mRNAs whose protein synthesis could be modulated through activity-dependent control of RISC. Interestingly, we also observed that one such novel RISC-regulated mRNA, Lysophospholipase 1 (Lypla1), is regulated by dendritically localized miR-138. Furthermore, using a translation reporter, we were able to visualize proteasomal degradation of MOV10 as a critical regulatory step for localized protein synthesis from synaptic mRNAs, such as Lypla1. Significantly, Lypla1 is a depalmitoylating enzyme that has been shown to remove the palmitate group from a cytoskeleton regulator, G_{α} (Duncan and Gilman, 1998); in addition, palmitoylation (a reversible posttranslational modification) has recently been identified as a key regulatory switch for synaptic plasticity (Kang et al., 2008).

Our observations, together with those of another study regarding miRNA-mediated control of long-term olfactory memory (Ashraf et al., 2006), set the stage for further study whose goal is to obtain a comprehensive view of regulatory control over synaptic plasticity. Next we discuss the methodologies used to identify synaptic mRNAs that are regulated by RISC and translated locally within the synaptodendritic compartment.

Translational Trap: A Novel Approach to Identifying Biological Targets of miRNAs

To gain insight into miRNA-mediated regulatory control of synaptic plasticity, it is important to know

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the entire set of synaptic mRNAs that miRNAs target. miRNAs bind to the 3′UTR region of their target mRNAs through imperfect base pairing. This imperfect base-pairing rule provides a significant challenge to identifying biological targets using *in silico* sequence analysis. However, nucleotides 2–8 in the miRNA (also known as the "seed region") have been shown to hybridize to an mRNA target through perfectly complementary base pairing. This seed-pairing rule is critical for appropriate target recognition (John et al., 2004; Lewis et al., 2005).

Based on this rule and other hybridization properties (e.g., free energy of duplex formation, accessibility of target site, and neighboring sequence around the miRNA binding site), several target prediction algorithms have been generated. All these target prediction tools provide a list of large numbers of putative mRNA targets for a single miRNA, and there is little overlap among target sets of specific miRNAs. Although the majority of miRNA-mediated neuronal gene expression control occurs at the translational level, miRNA–target interaction also has been shown to regulate gene expression through mRNA degradation (Kosik, 2006). However, target prediction algorithms do not account for the mode of regulatory control. In addition to these challenges, *in silico* target prediction tools do not assess the effect of cellular factors on miRNA–target interaction.

To overcome the limitations of target prediction tools and to identify biological targets of miRNAs, we designed a novel method we named "Translational Trap" (Banerjee et al., 2009). It has been shown that the core components of RISC play a critical functional role. One such novel core factor is MOV10, a DEAD box helicase and ortholog of the *Drosophila* homolog Armitage that has been implicated in miRNA-guided translational control (Cook et al., 2004; Chendrimada et al., 2007). Interestingly, we also observed that MOV10 is rapidly degraded by the proteasome in response to neuronal activity. Based on these observations, we reasoned that the removal of MOV10 could relieve RISC-mediated translational suppression, after which RISC-associated mRNAs would then be redistributed from the translationally suppressed pool to an actively translating fraction (Fig. 1).

The Translational Trap method takes advantage of short hairpin (sh) RNA–mediated RNAi against MOV10. We used a lentiviral delivery system for efficient RNAi knockdown because the efficacy of transfection methods in primary neuronal culture is very low. In order to visualize lentivirus-infected neurons, we cloned shRNA constructs in a lentiviral vector (pLVTHM) that also express EF1α promoter–driven enhanced green fluorescent protein (eGFP). Recombinant lentivirus was made by cotransfecting transfer vector (pLVTHM containing shRNAs against MOV10), packaging plasmid (psPAX2)–expressing viral packaging proteins, and envelope plasmid (pMD 2.G)–expressing viral coat proteins into HEK293T cells. The transfection was performed using the calcium phosphate method. Following transfection, we collected recombinant lentivirus from culture supernatant and viral titer analyzed by fluorescent-activated cell sorter (FACS) analysis. Primary neuronal culture at day *in vitro* (DIV) 7 was then infected with the recombinant lentivirus expressing shRNA against MOV10 at a multiplicity of infection (MOI) of 4. Following virus transduction,

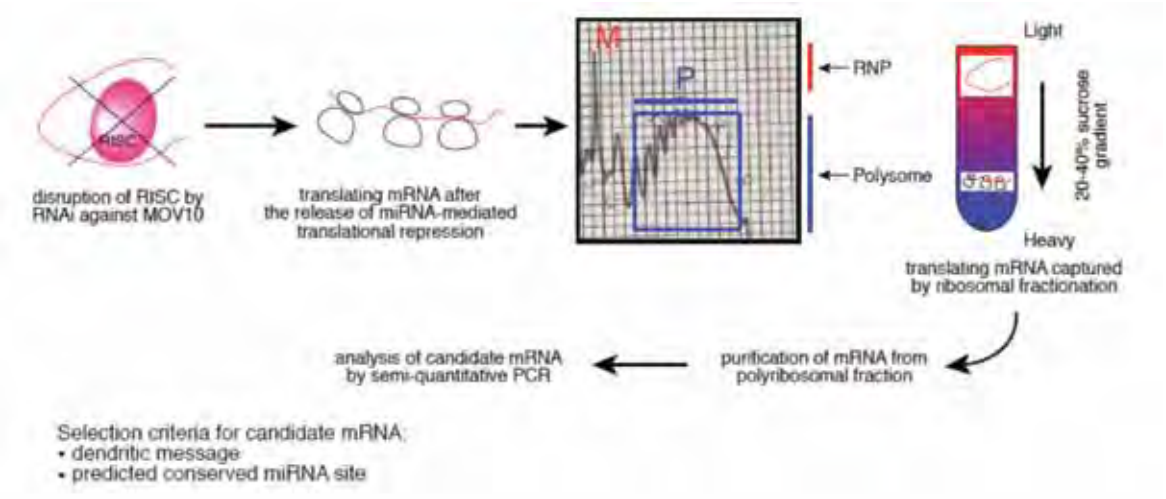

Figure 1. Schematic diagram of Translational Trap method.

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RNAi-mediated knockdown of MOV10 was tested at various time points. After obtaining more than 70% knockdown of MOV10 level at DIV18-19, neurons were used for polyribosomal fractionation.

We isolated polyribosomal pool from transduced neuronal culture using sucrose gradient fractionation, as described previously (Stefani et al., 2004), with minor modifications. We next arrested translation of mRNAs in primary neuronal cultures by washing them with ice-cold phosphate buffer saline (PBS) containing the protein synthesis inhibitor cycloheximide (15 μg/ml). Neurons were then homogenized and the cytoplasmic extract obtained by differential centrifugation. The cytoplasmic extract was then loaded onto 20–50% w/w linear density gradient of sucrose. The gradient was centrifuged at 40,000 × *g* for 2 h at 4°C in a SW41 rotor (Beckman Instruments, Fullerton, CA). Following centrifugation, the gradient was fractionated with continuous monitoring absorbance at 254 nM using a UA-6 ultraviolet (UV) detector (Teredyne ISCO, Lincoln, NE). Polyribosomal fractions were pooled, denatured by 1% SDS, and total RNA obtained via the precipitation method. The crude RNA fraction was further purified by using an Absolute RNA reverse transcriptase PCR (RT-PCR) Miniprep kit (Stratagene, La Jolla, CA).

To visualize mRNA distribution in polyribosmal fractions, 4 μg of total RNA was reverse-transcribed into double-stranded cDNA using a First Strand SuperScript II kit (Invitrogen, Carlsbad, CA) and analyzed by qPCR (quantitative polymerase chain reaction) using primer specific for 54 dendritic mRNAs. These mRNAs were chosen based on their localization (Poon et al., 2006; Zhong et al., 2006) and predicted conserved miRNA binding sites. qPCR was performed using SYBR Green–containing PCR amplification (Applied Biosystems, Carlsbad, CA). The 7500 Fast System Sequence Detection Software (Applied Biosystems) was used to analyze real-time PCR data, and the threshold cycle chosen in the linear amplification range of all samples. The qPCR data were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (rRNA) as internal standard. After normalization, the differential distribution of candidate mRNAs was assessed using the comparative C_T method (Schmittgen and Livak, 2008).

Our Translational Trap method revealed that miRNAs can potentially regulate 13 candidate mRNAs out of 54 dendritic messages. Interestingly, we also observed that some dendritic mRNAs, such as β-actin, as well as translation initiation factor Eif3s10, are not differentially redistributed after RNAi occurs against MOV10. Among several significantly redistributed candidates, Lypla1 and αCaMKII were studied further. Results from qPCR analysis were further verified by Western blot analysis of endogenous protein level after MOV10 RNAi took place.

Go Fishing: Identifying miRNA That Binds to RISC-Regulated mRNA

After identifying RISC-regulated mRNAs, our next challenge was to identify specific miRNAs that can hybridize to these mRNAs. To visualize such specific miRNA–target interaction, we relied on *in silico* target prediction tools. We first obtained a list of putative miRNAs that multiple algorithms predict will target a specific mRNA. For example, three major target prediction algorithms (TargetScan, PicTar, and miRanda) identified miR-138 as a potential miRNA that can bind to Lypla1. To assess the direct miRNA-target interaction, we performed a reporter assay after a loss-of-function experiment using lock nucleic acid (LNA) inhibitor (Orom et al., 2006) for a specific miRNA. The major advantage of LNA miRNA inhibitor is that it can efficiently and irreversibly bind to complementary miRNA and thus prevent it from functioning.

In order to perform the reporter assay, we fused the full-length 3′UTR of Lypla1 mRNA with Luciferase reporter. We then cotransfected this reporter construct with LNA inhibitor of miR-138 into primary hippocampal neurons at DIV7. We also cotransfected LNA scramble control in order to analyze the specificity of the reporter assay. We analyzed the effect of miR-138 on reporter expression by measuring Luciferase activity at 48 h after transfection. We observed that inhibition of miR-138 function leads to a significant increase in reporter activity, suggesting a direct interaction between miR-138 and Lypla1 mRNA. We further verified the results from the reporter assay by assessing endogenous protein level after similar inhibition of miR-138 function.

In order to assess miR-138–Lypla1 3′UTR interaction–mediated control of *de novo* protein synthesis at synapses, we first tested the localization of both miR-138 and Lypla1 mRNA using double-label *in situ* hybridization. We hybridized primary hippocampal neurons using an LNA antisense probe (Exiqon, Woburn, MA). We labeled miR-138 and Lypla1 with biotin and digoxigenin (DIG), respectively, using a DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Applied Science, Indianapolis, IN). We

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also used a scramble probe to assess the specificity of hybridization. *In situ* hybridization was performed with biotin or DIG-labeled antisense probes.

Following hybridization, neurons were washed extensively and incubated with cy3-labeled anti-DIG as well as fluorescein isothiocyanate (FITC)–labeled anti-biotin (Jackson ImmunoResearch, West Grove, PA). To visualize synaptic localization, neurons were immunostained with antibody specific to Synapsin I, a synaptic marker, followed by incubation with cy5-conjugated secondary antibody. After immunostaining, confocal microscopy was used to visualize synaptic localization of both miR-138 and Lypla1 mRNA (Fig. 2*A*). Similarly, our *in situ* hybridization procedure, combined with immunostaining, showed that MOV10, a critical component of RISC, colocalizes with Lypla1 mRNA (Fig. 2*B*). These observations suggest that Lypla1 is an ideal candidate for studying the activity-dependent control of RISC-mediated localized protein synthesis that occurs at the synapse.

RISCy Control of Synaptic Protein Synthesis: An Online Approach

After we observed that neuronal activity can lead to rapid synaptic degradation of endogenous MOV10 through NMDA receptor activation, we designed an imaging-based method for quantitatively assessing *de novo* degradation of MOV10 in living hippocampal neurons. For this purpose, we fused a photoactivable green fluorescent protein (PA-GFP) with MOV10 (Patterson and Lippincott-Schwartz, 2002) and expressed the fusion protein in hippocampal neurons using lentivirus transduction. Similar to endogenous MOV10, when imaged, ectopically expressed MOV10-PA-GFP appeared as a punctate pattern. The unique photoactivation property of PA-GFP provides an advantage when scoring localized protein degradation, as any new synthesis of MOV10 will not affect our analysis.

We proceeded to photoactivate MOV10-PA-GFP–expressing neurons using a 365 nm UV lamp and stimulated synaptic activity in these neurons (DIV21-24) with the membrane depolarizing agent KCl (60 mM for 5 min). We used confocal microscopy to capture images at specific time intervals (Fig. 3*A*). We then analyzed time-lapse images to compute MOV10 puncta intensity over time using a custom-written algorithm in MATLAB (MathWorks, Natick, MA). Similar to endogenous MOV10 degradation, we observed that the RISC protein MOV10 is degraded rapidly at synaptodendritic compartments in response to neuronal activity (Fig. 3*B*). Interestingly, we also observed that the rapid degradation of MOV10-PA-GFP was prevented when neurons were pretreated with the proteasome inhibitor lactacystin. These

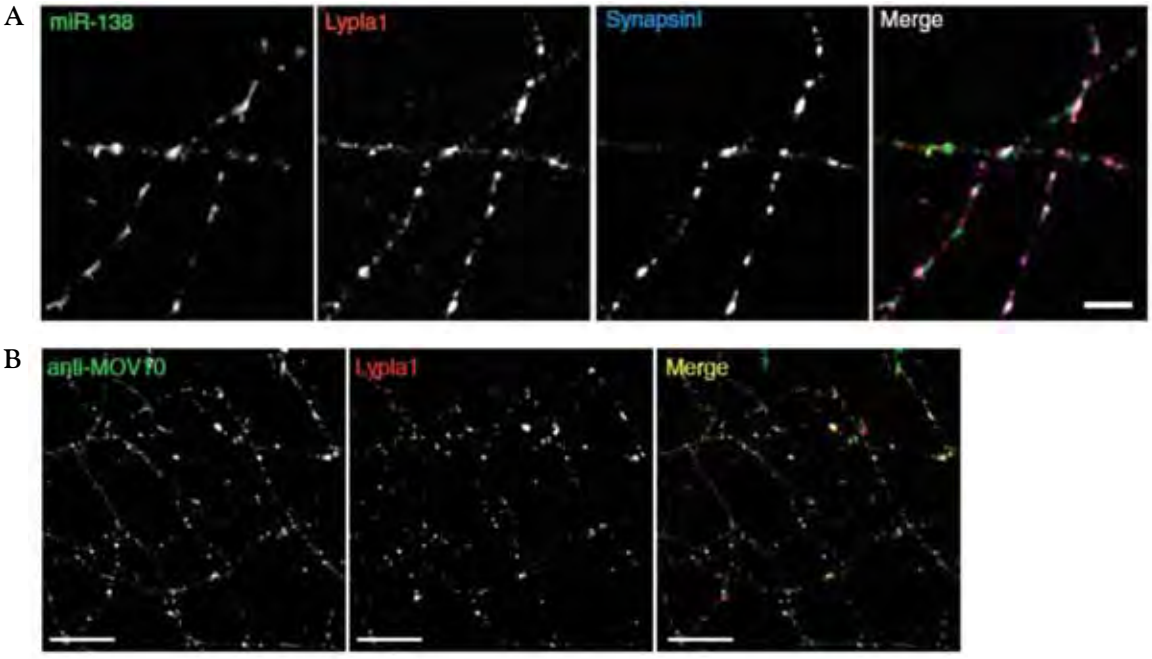

Figure 2. *In situ* hybridization showing localization of miR-138 and Lypla1 mRNA. ***A***, Double *in situ* hybridization to visualize synaptic localization of miR-138 and Lypla1 mRNA. Scale bar, 10 μm. ***B***, Lypla1 mRNA is associated with the RISC component MOV10. Scale bar, 20 μm.

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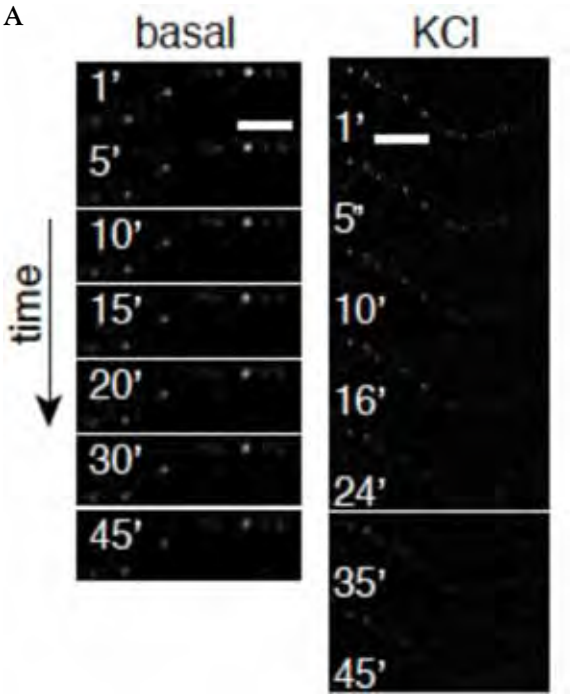
observations indicate that rapid synaptic degradation of MOV10 occurs through proteasomal control. We also analyzed the diffusion properties of MOV10-PA-GFP and observed that the ectopically expressed protein was not freely diffusing but rather bound to a restricted space. This observation further confirmed that the decrease in puncta intensity occurred as a result of degradation of ectopically expressed protein rather than its free diffusion elsewhere.



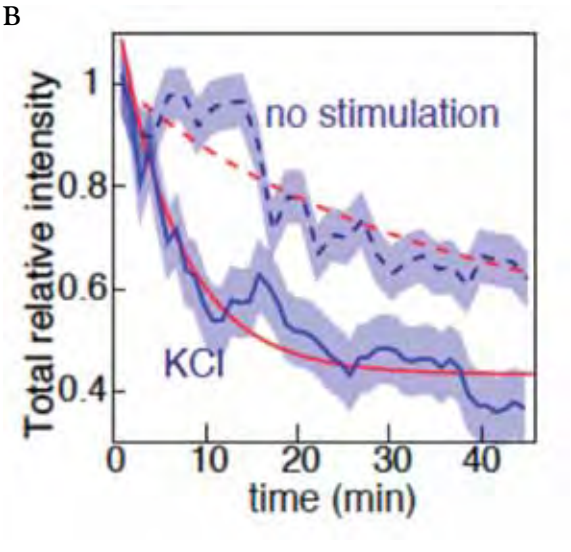


Figure 3. Activity-dependent degradation of MOV10-PA-GFP. ***A***, Time-lapse image of MOV10-PA-GFP–expressing neurons with or without synaptic activation. Scale bar, 10 μm. ***B***, Quantitative profile of MOV10-PA-GFP level with or without stimulation.

To address how activity-dependent control of RISC can regulate dendritic protein synthesis, we took an imaging approach to scoring localized protein synthesis using the photoconvertible reporter Kaede (Ando et al., 2002). The green form of Kaede can be converted to the red form by 365 nm UV light. This photoconversion is efficient, rapid, and irreversible. Kaede's unique photoconversion property offers a tremendous advantage for marking the existing protein and scoring newly synthesized protein following synaptic activation. Thus, we fused Lypla1 3′UTR with Kaede reporter and expressed it in cultured hippocampal neurons using lentivirus transduction. Kaede reporter appears as a punctate pattern because Kaede is a tetrameric protein that can form aggregates even at low concentration; aggregate formation can be enhanced further because of its expression in restricted space, such as a dendritic spine.

Following photoconversion, we stimulated hippocampal neurons (DIV21-24) using membrane depolarization (60 mM KCl for 5 min) or glutamate treatment (20 μM for 5 min). After stimulation, we captured time-lapse images in the green channel to score any new protein synthesis, and in the red channel, to monitor changes in dendritic spine volume (Tanaka et al., 2008). The quantitative assessment of new protein synthesis was performed by a custom-written algorithm in MATLAB (MathWorks). Our imaging analysis showed that Lypla1 3′UTR–driven localized translation of Kaede occurs in response to synaptic activity (Fig. 4*A*). This activity-dependent translation was inhibited when neurons were pretreated with the proteasome inhibitor lactacystin, and the protein-synthesis inhibitor anisomycin. However, *de novo* protein synthesis was not prevented by pretreatment with the transcription inhibitor actinomycin D. Taken together, these observations clearly suggest that Lypla1 3′UTR–driven localized control of dendritic protein synthesis is also regulated by proteasomal control, but not through transcriptional mechanisms (Fig. 4*B*).

Because Lypla1 3′UTR duplexed with miR-138 modulates localized translation through proteasomal control, we performed similar imaging analysis to visualize the role that the key RISC component, MOV10, plays in regulating dendritic protein synthesis. To further apply this approach, we first reduced the endogenous level of MOV10 by RNAi and then stimulated these neurons with glutamate. We observed that reducing the level of endogenous MOV10 completely blocked Lypla1 3′UTR–driven new protein synthesis of translation reporter

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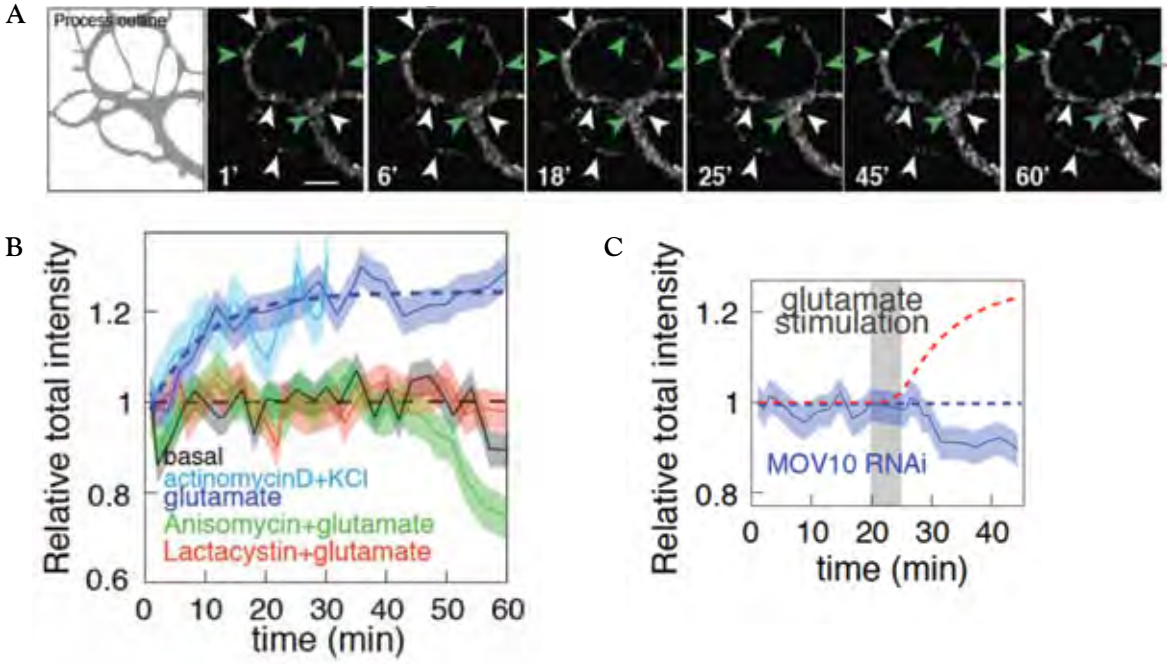

Figure 4. RISC-mediated degradative control of synaptic protein synthesis. ***A***, Lypla1 3′UTR–driven, activity-dependent localized control of protein synthesis. Scale bar, 10 μm. ***B***, Quantitative profile of newly synthesized translation reporter with or without glutamate stimulation. *De novo* protein synthesis of translation reporter is inhibited by the proteasome inhibitor lactacystin and the translation inhibitor anisomycin but not by the transcription inhibitor actinomycin D. ***C***, Lypla1 3′UTR–driven localized protein synthesis is completely blocked by RNAi-mediated knockdown of MOV10.

(Fig. 4C). This observation set the stage for analyzing whether MOV10 degradation and Lypla1 3′UTR–driven localized synthesis occurs in a coordinated manner.

To visualize the direct link between these two biochemical processes, we cotransduced hippocampal neuron with lentivirus-expressing cyan fluorescent protein (CFP)–fused MOV10 (MOV10-CFP) and Lypla1 3′UTR–fused Kaede (Fig. 5*A*). After photoconversion, we analyzed MOV10 degradation by following CFP intensity as well as new protein synthesis by measuring the level of green-form Kaede in the same punctum. Quantitative analysis of time-lapse images revealed that 35% of puncta showed a strong correlation between localized protein degradation and synthesis (Fig. 5*B*). Our observation was also supported by double-label *in situ* hybridization data showing that a similar percentage of puncta contains both endogenous Lypla1 mRNA and miR-138. Furthermore, an increase in MOV10 degradation directly correlated with the

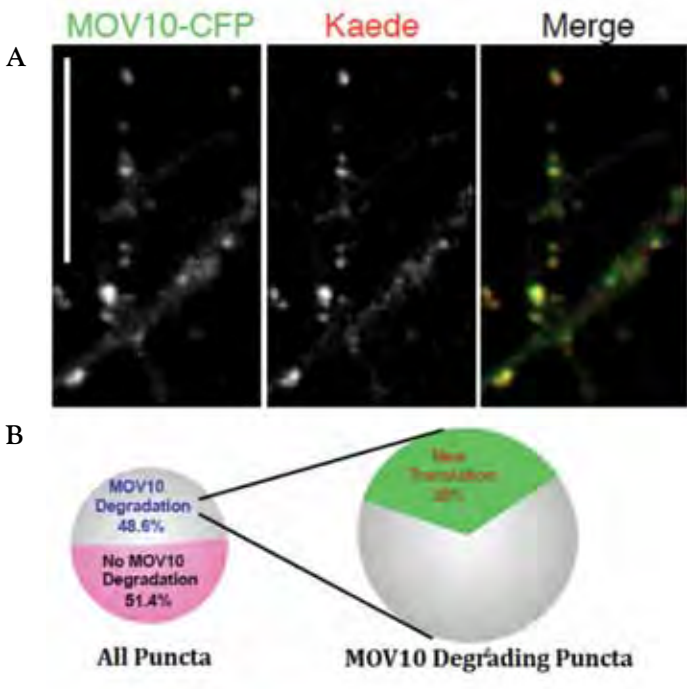

Figure 5. Degradative control of RISC is directly linked with localized protein synthesis from a specific class of mRNAs. ***A***, Localization of ectopically expressed MOV10 and Lypla1 3′UTR–fused Kaede. Scale bar, 20 μm. ***B***, MOV10 degradation and Lypla1 3′UTR–driven localized protein synthesis occur at the same location.

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enhancement of *de novo* protein synthesis. As further support, a related recent study showed that miR-138–mediated control of Lypla1 (also known as APT1) expression can modulate synaptic development (Siegel et al., 2009). These observations suggest that activity-dependent control of synaptic modification can be regulated by this novel type of posttranslational modification.

Conclusion

A comprehensive understanding of miRNA-mediated posttranscriptional control of synaptic function has just begun. Towards this end, our recent analysis established a novel cell-biological and biochemical approach to understanding miRNA-mediated regulation of synaptic plasticity. However, a holistic view of miRNA-mediated control of long-term memory formation through this type of novel plasticity mechanism remains relatively unexplored. Future experiments will need to incorporate new methodologies for visualizing miRNA-mediated control over synaptic protein synthesis in a living animal during memory acquisition. Insight into these novel memory mechanisms could eventually lead to the discovery of new therapeutic strategies for a variety of neurodegenerative diseases, such as Alzheimer's disease, as well as neurological conditions such as autism and mental retardation.

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Strategies for Exploring Local Dendritic Protein Synthesis in Synaptic Plasticity and Memory

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Introduction

Protein biosynthesis plays an essential role in controlling virtually every aspect of eukaryotic cell function. Work over the last several decades in highly tractable model systems, such as yeast, has revealed an intricately complex mechanism for mRNA translation that is subject to powerful regulation by diverse signaling pathways at almost every stage. In more complex biological networks, the functional impact of protein synthesis has also been shown to be under powerful spatial control, particularly in highly polarized cells such as neurons. In these networks, axons and dendrites extend over great distances from the neuronal cell body, suggesting that local translation in these distinct compartments may be critical to maintaining the unique functional specialization of these areas. Indeed, local protein synthesis in axonal growth cones and dendrites is now known to play a diverse repertoire of functional roles, both in the initial wiring of synaptic connectivity during development (Campbell and Holt, 2001; Brittis et al., 2002) and in the maintenance (Sutton et al., 2006) and plasticity (Kang and Schuman, 1996; Huber et al., 2000) of those connections, once formed.

Moreover, evidence continues to link loss of this local translational control with a growing list of human disorders characterized by cognitive dysfunction, including fragile X syndrome and autism (Kelleher and Bear, 2008; Wang et al., 2010). The fact that altered local translational control in neurons is associated with profound cognitive deficits draws an interesting parallel with the well-known role of protein synthesis in the formation of long-term memories (Flexner et al., 1963; Agranoff et al., 1965). However, it has not yet been possible to establish a definitive role for local protein synthesis at synapses in memory processing.

A major hurdle stands in the way of defining the specific roles that local protein synthesis plays in synaptic function and memory: the inherent challenge of specifically manipulating translation efficiency in distinct cellular compartments. This chapter summarizes the main approaches that have been used to tackle this problem. The main purpose of this work is to focus on the techniques themselves and what principles these techniques have served to demonstrate as well as their existing limitations, rather than to summarize an overall picture of the functional capabilities of local translation. To gain a deeper appreciation of the biological roles of compartmentalized protein synthesis in neurons, the reader is directed to several recent reviews (Sutton and Schuman, 2006; Costa-Mattioli et al., 2009; Wang et al., 2010).

Approaches to Visualizing Local Protein Synthesis

Transmission electron microscopy

Visualization methods provided the initial evidence (and for many years, the only evidence) that dendrites are capable of autonomous translational control. Using transmission electron microscopy (TEM), Bodian (1965) observed apparent ribosomes in proximal dendrites of monkey spinal cord neurons, where they were found close to sites of synaptic contact. His observations prompted him to speculate that local translation beneath these synapses might function to establish and modify synaptic connectivity. Several years later, Steward and Levy (1982) demonstrated that polyribosomes could be detected in the distal dendrites of dentate granule cells of the hippocampus, where they tend to cluster beneath sites of excitatory synaptic contact (beneath dendritic spines). Their observation of dendritic polysomes, suggesting active dendritic translation so remote from the neuron cell body, provided much stronger evidence for a role of local translation in regulating synaptic function.

TEM, owing to its unique ability to resolve the fine intracellular structure of neurons, has remained an important tool for understanding local protein synthesis in dendrites and its relationship to synaptic plasticity and memory. For example, Harris and colleagues have used TEM-guided detection of polyribosomes, following synaptic plasticity induction in hippocampal slices, to document a translocation of polyribosomes from dendritic shafts into spines of CA1 pyramidal neurons during long-term potentiation (LTP) (Ostroff et al., 2002). Although this approach is labor-intensive, and before-and-after comparisons in the same preparation are not possible, it does offer the opportunity to delve into questions regarding local translational regulation during memory processing. In a recent study, for example, Ostroff et al. (2010) demonstrated that fear conditioning increases both the number of dendritic polyribosomes and their association with the spine apparatus in lateral amygdala neurons. The observed increase in polyribosomes likely reflects an overall increase in dendritic translation after fear learning, although other interpretations cannot be fully ruled out.

Fluorescent protein–based translation reporters

The major limitation of TEM is that changes in dendritic translation relative to baseline cannot be studied in the same sample. This deficiency prompted the development of methods to dynamically visualize

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local synthesis of proteins in neuronal processes. The first such tools developed used a diffusion-limited fluorescent translation reporter in which a region encoding a destabilized, myristoylated green fluorescent protein (GFP) is flanked by the 5′ and 3′ untranslated regions (UTRs) from αCaMKII mRNA (Aakalu et al., 2001), an mRNA known to be dendritically localized. The half-life of this destabilized GFP is >90 min, and the myristoylation tag has been shown to severely inhibit the free diffusion of the reporter once made (Aakalu et al., 2001).

This translation reporter has been used to examine both activity-dependent (Sutton et al., 2004) and neuromodulatory control (Aakalu et al., 2001; Smith et al., 2004) of dendritic protein synthesis in hippocampal neurons. In addition, transgenic expression of a similar reporter (yellow fluorescent protein [YFP] fused to the 3′ UTR of CaMKII) allowed Ashraf et al. (2006) to examine changes in dendritic reporter repression in defined glomeruli of the *Drosophila* antennal lobe after an associative olfactory conditioning task. Importantly, this particular translation reporter served as proof-of-principle for the design strategy itself, which encouraged the development of new reporters with improved characteristics.

One of the major improvements in this class of translation reporter has come from replacing a generic fluorescent protein with either photoconvertible fluorescent proteins (e.g., Kaede or Dendra) or epitope tags that bind spectrally distinct fluorescent dyes with high affinity (e.g., FlAsH/ReAsH). These modifications make it possible to distinguish newly synthesized reporter from preexisting fluorescent signal. Also, they allow for a more precise "dating" of new reporter signal without the need for photobleaching existing signal. These newer reporter systems have been used to examine local synthesis of sensorin at *Aplysia* synapses (Wang et al., 2009) and local dendritic synthesis of AMPA receptor subunits GluA1 and GluA2 (Ju et al., 2004) as well as Kv1.1 voltage-gated potassium channels (Raab-Graham et al., 2006).

TimeSTAMP

Recently, Tsien and colleagues introduced a novel strategy for monitoring new synthesis of candidate proteins (Lin et al., 2008). This approach, termed time-specific tagging for the age measurement of proteins (TimeSTAMP), utilizes fusion of the hepatitis C virus (HCV) protease upstream of its cognate recognition sites surrounding an epitope/fluorescent protein tag introduced into a protein of interest. The resulting default *cis* cleavage by HCV protease results in constitutive removal of the visualization tag upon synthesis. Hence, under basal conditions, newly synthesized proteins are not fluorescent. By coupling this strategy with a small-molecule HCV protease inhibitor (to prevent proteolysis of the fluorescent tag), new synthesis of the resulting protein can be visualized with little or no background from preexisting protein. Applying this twofold strategy, Lin et al. (2008) were able to demonstrate that newly synthesized recombinant PSD95 and CaMKII could be visualized in cultured neurons and intact fly brains, respectively. Moreover, the shift in molecular weight that protease cleavage confers provides a useful signature for distinguishing newly synthesized and preexisting proteins in Western blots, illustrating that the usefulness of TimeSTAMP extends beyond visualization.

F2P fluorescence labeling

The reporter strategies outlined above all require a candidate-based approach. Thus, these reporters are overexpressed in neurons and are not likely to reveal endogenous translational control principles influenced by mRNA availability, which could be circumvented by overexpression. A related limitation is that these candidate-based reporters do not yield a complete picture of local translation beyond the specific candidate in question.

Novel reporter systems developed during the last few years have used a modified design that fluorescently labels endogenous proteins as they are synthesized. The first of these, a fluorescein-conjugated derivative of puromycin (F2P), exploits the fact that puromycin is a tRNA-mimetic that becomes incorporated into elongating polypeptides during protein synthesis. Smith et al. (2004) utilized bath and focal application of F2P to dendrites in order to demonstrate that dopamine D_1/D_5 receptor activation potently drives overall protein synthesis in dendrites. Importantly, F2P fluorescence was substantially reduced (though not eliminated entirely) by co-applying protein synthesis inhibitors. This effect indicates that the majority of detected signal reflected F2P integration into endogenous proteins. However, because F2P is intrinsically fluorescent, conditions need to be optimized in order to minimize the influence of unincorporated F2P. Moreover, although it is a useful reporter strategy, the impact of F2P incorporation on endogenous protein function is difficult to gauge, making it less desirable for functional studies.

FUNCAT

A slightly different approach, recently developed by Dietrich and colleagues (2010), uses an inventive

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strategy of allowing neurons to take up noncanonical amino acids with novel chemical properties, which are then exploited to label newly synthesized proteins that have incorporated them. This strategy, termed fluorescence noncanonical amino acid tagging (FUNCAT), has been successfully used for a variety of tasks: to visualize dendritic protein synthesis, to monitor the fate of proteins synthesized in distinct cellular compartments, and to visualize the mobility of newly synthesized cell surface proteins. A major advantage of FUNCAT is its ability to generate views of the complete translational response a particular set of conditions induces; a disadvantage is that it yields little information about the new synthesis of specific proteins. Hence, newer technologies such as TimeSTAMP and FUNCAT are complementary techniques and should be chosen based on the specific experimental question in mind.

Limitations of current visualization methods

One important limitation of visualization methods, as utilized, is that they provide information that is correlative in nature. Thus, although these methods fill a critical gap in our understanding of activity-dependent and neuromodulatory-dependent control of local translation, they do not reveal whether such local synthetic events actually contribute to changes in synaptic function. Below, I describe alternative approaches that have been applied specifically with this limitation in mind.

In vitro Approaches to Link Local Protein Synthesis with Synaptic Plasticity

Physical isolation methods

Physically separating the neuronal processes from their somata by way of surgical cuts is one of the more definitive methods for ensuring that the source of proteins required for synaptic plasticity derives from a local dendritic pool. This method has been used in cultured neurons (Aakalu et al., 2001; Ju et al., 2004) but is particularly useful in hippocampal slices. In the hippocampus, pyramidal neurons cluster in a defined layer and extend apical dendrites in a characteristic orientation through the laminar regions of synaptic neuropil. Surgically isolating dendritic lamina from parent cell bodies is thus easily accomplished using a dissection microscope, and synaptic responses from these isolated dendrites can be measured by taking field potential recordings with an extracellular electrode.

Kang and Schuman (1996) first applied this approach to the question of protein synthesis–dependent forms of synaptic plasticity. They found that isolated CA1 dendrites could still support translation-dependent synaptic potentiation induced by brain-derived neurotrophic factor (BDNF). Using a similar surgical technique, Huber and colleagues (2000) later demonstrated that long-term depression (LTD) induced by activation of group I metabotropic glutamate receptors (mGluR-LTD) also requires local dendritic protein synthesis. Other protein synthesis–dependent forms of synaptic plasticity have been similarly revealed using isolated dendritic laminae (Sutton and Schuman, 2006), and this preparation has been used in RNA profiling studies to estimate the population of mRNAs localized to dendrites (Zhong et al., 2006).

One important caveat with surgical isolation in hippocampal slices is that the dendritic lamina still contain interspersed interneurons and astrocytes. These particles could participate in the induction of synaptic plasticity (Stellwagen and Malenka, 2006), and their gene products are likely to be detected in RNA profiling studies. Hence, on its own, surgical isolation leaves open the possibility that protein synthesis in these cells, and not the dendrites of pyramidal neurons, is contributing to the observed outcome. Huber and colleagues (2000) nicely ruled out this possibility experimentally by demonstrating that translation in the pyramidal neurons is required during mGluR-LTD. Still, the "contaminating" influence of nonpyramidal cells in isolated dendritic lamina remains a limitation of the surgical isolation approach overall.

Synapse-enriched biochemical fractions

The use of biochemical fractions enriched in synaptic structures has remained a popular method for examining local translational regulation at synapses. Several variants of these fractions are routinely used, including synaptosomes, synaptoneurosomes, and synaptodendrosomes. Of these, synaptoneurosomes have been the most commonly employed. These structures contain presynaptic terminals and resealed postsynaptic compartments that remain physically associated and thus have a characteristic "snowman" appearance in TEM micrographs (Muddashetty et al., 2007). These fractions have been used to estimate synaptic mRNA content (Chicurel et al., 1993) and broad translational activation at synapses (Weiler and Greenough, 1993; Takei et al., 2004) as well as to monitor the *de novo* synthesis of specific proteins such as the fragile X mental retardation protein (FMRP) (Weiler et al., 1997); GluA1 (Muddashetty et al., 2007; Aoto et al., 2008); Arc (Yin et al., 2002; Waung et al., 2008); and many others.

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A major advantage of biochemical fraction preparation is that it allows one to apply conventional biochemical techniques (e.g., Western blotting, metabolic labeling) and more recently developed proteomic approaches to the investigation of local translational control at synapses. Indeed, a promising future application of these preparations will be to use them in conjunction with newly developed methods of purifying newly synthesized protein pools (e.g., BONCAT [bio-orthogonal noncanonical amino acid tagging]) (Dietrich et al., 2006) in order to estimate the complement of proteins that may be locally synthesized at synapses. However, this approach also has several limitations, including concerns over the "purity" of synaptic components in these fractions and the fact that, once isolated, these fractions do not capture potentially important trafficking events to and from synaptic compartments that might powerfully regulate protein synthesis at synapses (Ostroff et al., 2002).

Microperfusion techniques

In recent years, spatially restricted microperfusion approaches have been powerful tools for linking local protein synthesis in dendrites with the induction of specific synaptic adaptations associated with altered neurotransmission. The first successful application of this general approach to studying compartmentalized protein synthesis during synaptic plasticity used the sensory neuron to motor neuron (SN-MN) synapse of *Aplysia* as a model system.

Martin and colleagues (1997) took advantage of a cell culture system in which a single SN makes separate synaptic contacts with two MNs, thus allowing for treatments to be selectively restricted to one SN-MN contact. Using local pressure ejection of serotonin (5-HT) from a micropipette, coupled with fast-bulk perfusion for rapid clearance, this group showed that local application of 5-HT to the site of synaptic contact induced both short-term facilitation (STF) and long-term facilitation (LTF) of synapses at those inputs without altering SN-MN function at the other SN-MN contact. Importantly, including protein synthesis inhibitors in the puffer pipette completely prevented LTF induced by repeated 5-HT pulses but without altering STF induced by a single 5-HT pulse. These results clearly demonstrated a crucial role for local protein synthesis in the SN terminal in inducing long-lasting synaptic facilitation. This unique SN-MN culture system has been a powerful tool for both identifying locally translated proteins that contribute to LTF (Wang et al., 2009) and defining how these products confer unique functional abilities on the synapses that synthesize them (Casadio et al., 1999; Si et al., 2003; Miniaci et al., 2008).

Local microperfusion has also been adapted to study the functional impact local translation has in mammalian neurons. Typically, this approach is carried out in monolayers of neurons in primary culture, which affords the ability of targeting treatments to specific regions of a neuron of interest through a dual micropipette perfusion system. In hippocampal neurons, dendrites are highly sensitive to mechanical effects (M. A. Sutton, unpublished observations), making pressure ejection through a "puffer" pipette a less desirable strategy. Instead, a delivery micropipette is fastened to allow the perfusate to escape under conditions of very-low-positive pressure, and a nearby suction pipette is positioned to draw a stream of perfusate across areas of interest and to remove the perfusate from the bath. This dual micropipette system is also typically used with a bulk flow perfusion oriented away from the neuron of interest. A fluorescent dye is included in the delivery pipette to monitor the size and stability of the perfusion region over the course of an experiment.

Although it is challenging to maintain a stable local perfusion region over the course of 1-2 h, several groups have successfully applied this approach to examine local regulation of protein synthesis reporters (Smith et al., 2004; Sutton et al., 2007; Dietrich et al., 2010); study the expression of target proteins thought to be locally synthesized (Sutton et al., 2006; Waung et al., 2008); and for other applications (Ibata et al., 2008). When used to locally deliver protein synthesis inhibitors, the primary advantage of local microperfusion is that the contribution of local translation can be assessed in intact neurons in which a somatic source of proteins is still available. Hence, by targeting protein synthesis inhibitors to either the dendrites or cell body (Dietrich et al., 2010), unique roles for translation in each compartment can be assessed under the same set of experimental conditions.

A particularly promising extension of the local microperfusion approach has come with the implementation of microfluidic chambers that can be used to maintain different neuronal compartments in distinct microenvironments. In a recent paper, Taylor and colleagues (2010) designed a novel microfluidic chamber with a local perfusion channel running perpendicular to the microgrooves in which dendrites and axons are localized but cell bodies are not. In order to validate their design, Taylor et al. were able to use these chambers to locally apply glutamate to synaptic regions in different patterns and to monitor cAMP response element-binding protein (CREB) phosphorylation in the cell soma.

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Microfluidic chambers remove many of the technical challenges associated with local microperfusion; thus, in principle, they allow for more extended, spatially restricted treatment applications as well as greater stability of the local perfusion area. These chambers are likely to be a valuable resource when studying local translation at synapses.

Optical approaches

Optical approaches offer perhaps the most exciting opportunities for designing novel methods of manipulating protein synthesis on fine spatial scales. One strategy typified by such approaches is to couple the use of caged protein synthesis inhibitors with focal ultraviolet (UV) light exposure to inhibit protein synthesis in a spatially tunable fashion.

Goard et al. (2005) developed the first of such compounds: dimethoxynitrobenzyl (DMNB)–Anisomycin and bromohydroxycoumarin (Bhc)–Anisomycin. They demonstrated (with Bhc-Anisomycin) that UV light exposure could impart spatially restricted inhibition of protein synthesis. More recently, Woolley and colleagues (Sadovski et al., 2010) greatly expanded the repertoire of photoreleasable translation inhibitors by introducing a diethylaminocoumarin (DEAC) caged version of anisomycin (DEAC-Anisomycin) as well as caged 4E-BP peptides and caged rapamycin. These new caged compounds will allow not only for spatially targeted inhibition of general protein synthesis (with caged anisomycin) but also for more selective inhibition of cap-dependent translation (with caged 4E-BP) and mammalian target of rapamycin complex 1 (mTORC1)–driven protein synthesis (caged rapamycin).

Because optical methods permit precise spatial and temporal control, these compounds have obvious potential for inhibiting protein synthesis with high spatial resolution (potentially, at single synapses). However, the selective disruption of dendritic protein synthesis has yet to be shown with such caged inhibitors, although the effectiveness of microperfusing such inhibitors suggests that these are almost certainly effective for this purpose. Another limitation of optical approaches is that repeated UV light exposure has potential secondary consequences for neuronal physiology. This problem can be circumvented using two-photon excitation to photorelease, as shown by Goard et al. (2005). In addition, the DEAC-Anisomycin caged compound developed by Sadovski et al. (2010) has longer wavelength absorption and can be effectively uncaged with 405 nm light. This characteristic is predicted to have fewer secondary consequences and to make this tool useful to a wider community, given that 405 nm laser lines are now common on many commercial microscope systems.

In vivo Approaches to Link Local Protein Synthesis with Memory Processing

Given that many enduring forms of synaptic plasticity critically depend on local protein synthesis in the dendritic compartment, similar compartmentalized synthesis likely plays a fundamental role in memory processing. However, testing this general hypothesis has proven extremely difficult owing to the technical challenges of manipulating translation in a compartment-specific fashion *in vivo*. At this point, we still lack the appropriate tools to accomplish this goal, but there are indications that it is feasible in the long run.

One strategy to address this question was illustrated by Miller et al. (2002), who generated mutant mice in which the native 3′UTR of αCaMKII mRNA had been replaced with the 3′UTR of bovine growth hormone to prevent its dendritic localization. Whereas dendritic αCaMKII mRNA was completely abolished, the somatic αCaMKII mRNA pool was only slightly decreased relative to wild-type mice. These mice exhibited deficits in protein synthesis–dependent forms of LTP as well as memory in hippocampal-dependent learning tasks, suggesting a potential role for dendritic αCaMKII synthesis in LTP and learning.

Recently, a similar strategy was adopted for excluding BDNF mRNA from dendrites (An et al., 2008). BDNF mRNA transcripts contain one of two 3′UTRs: The expression of the short UTR is restricted to cell bodies, whereas the long UTR is trafficked to dendrites. An et al. utilized a mouse mutant in which the long 3′UTR is truncated; they found little BDNF mRNA in dendrites and that, despite normal overall levels of BDNF protein, dendritic BDNF levels were substantially diminished. Similar to the αCaMKII 3′UTR mutants (Miller et al., 2002), these animals showed deficits in protein synthesis–dependent forms of LTP, as well as altered spine morphology in CA1 pyramidal neurons.

Together, these studies suggest a potential role for dendritic protein synthesis in regulating synaptic plasticity and memory. However, because the modified mRNAs in these studies were present throughout development, it was not possible to rule out the notion that the constitutive loss of these proteins at synapses, rather than *de novo* synthesis, accounted for the deficits. Although the specific

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implications of these findings with respect to *de novo* dendritic synthesis of αCaMKII and BDNF remain uncertain, these studies are important because they make the initial inroads towards defining a role for local dendritic translation in long-term memory formation. Perhaps by combining this general strategy with conditional genetic, chemical genetic, or optogenetic tools, it may be possible to disentangle the roles of local *de novo* synthesis from basal depletion of dendritic protein expression.

Perspective

Our understanding of local translation in neurons has closely followed technical innovations that have provided new access for investigating questions about the activity-dependent control of dendritic protein synthesis and its impact on synaptic function in different contexts. We have learned a great deal in recent years about activity-dependent control of protein synthesis in dendrites and axons, as well as how such local translation contributes to synapse formation, maintenance, and plasticity. However, our ability to address some of the larger questions surrounding this field, such as what the compartmentalized roles of translation play in learning and memory, remain severely limited. A major challenge going forward is to develop innovative approaches, particularly *in vivo* strategies, to begin to address this fundamental question.

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MicroRNA Regulation of CNS Myelination

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Introduction

MicroRNAs (miRNAs) are a class of small (~22 nt) noncoding RNAs that are capable of posttranscriptionally silencing mRNAs that contain sequences complementary to the miRNAs' 7–8 bp "seed" sequence (Bartel, 2004; Wu and Belasco, 2008). Because single miRNAs are predicted to often target up to hundreds of individual transcripts, miRNAs are able to broadly affect the overall protein expression state of the cell. This capability can translate into global effects on cellular health and differentiation state. Recently, several reports have identified crucial roles for miRNAs in controlling the production, differentiation, and health of myelinating cells of the mammalian nervous system. In this chapter, we will discuss how individual miRNAs regulate these various processes and how miRNA production in general is required for several stages of myelin generation and maintenance.

Oligodendrocytes Require miRNAs at Various Stages of Development

To study the overall role of miRNAs in biological processes, researchers have knocked out enzymes required for normal miRNA processing, such as Dicer1 (Bartel, 2004). However, *Dicer1*$^{-/-}$ mice die embryonically (Bernstein et al., 2003). Therefore, to study the requirement of mature miRNAs in postnatal processes, Cre-mediated recombination has been used to disrupt Dicer1 function in specific cells of interest. In this way, functional miRNAs have been shown to be required at all stages of oligodendrocyte (OL) generation and myelination in the mammalian CNS.

The knockout of Dicer1 function in uncommitted neural precursors, by driving Cre expression from the *Nestin* promoter, leads to a reduction in overall OL lineage cell number: both mature OLs and immature OL precursor cells (OPCs) (Kawase-Koga et al., 2009). This effect may represent a reduction in OPC generation from neural precursors, as opposed to OPC proliferation, because disruption of Dicer1 function in specified OPCs and OLs, by expressing Cre from either the *Olig1* or *Olig2* promoter, does not reduce OPC number *in vivo* (Dugas et al., 2010; Zhao et al., 2010). However, OPCs that lack Dicer1 do fail to differentiate normally because OL differentiation and myelin formation are significantly disrupted in *Olig1-Cre*, *Olig2-Cre*, and *CNP1-Cre Dicer*$^{Flox/Flox}$ mice, and OPCs purified from these animals fail to differentiate normally *in vitro*. Finally, mature miRNA production is not only necessary during development but is also required to maintain healthy myelin: Disrupting Dicer1 function specifically in fully mature OLs, by driving tamoxifen-inducible Cre expression from a *PLP* promoter, leads to the eventual degradation of fully formed CNS myelin (Shin et al., 2009).

In summary, these results indicate that mature miRNA activity is required at various stages of OL development:

- In the initial production of fate-specified OPCs;
- In the differentiation of mature OLs and generation of compact CNS myelin during development; and
- In the maintenance of functional myelin sheaths in older animals (Fig. 1).

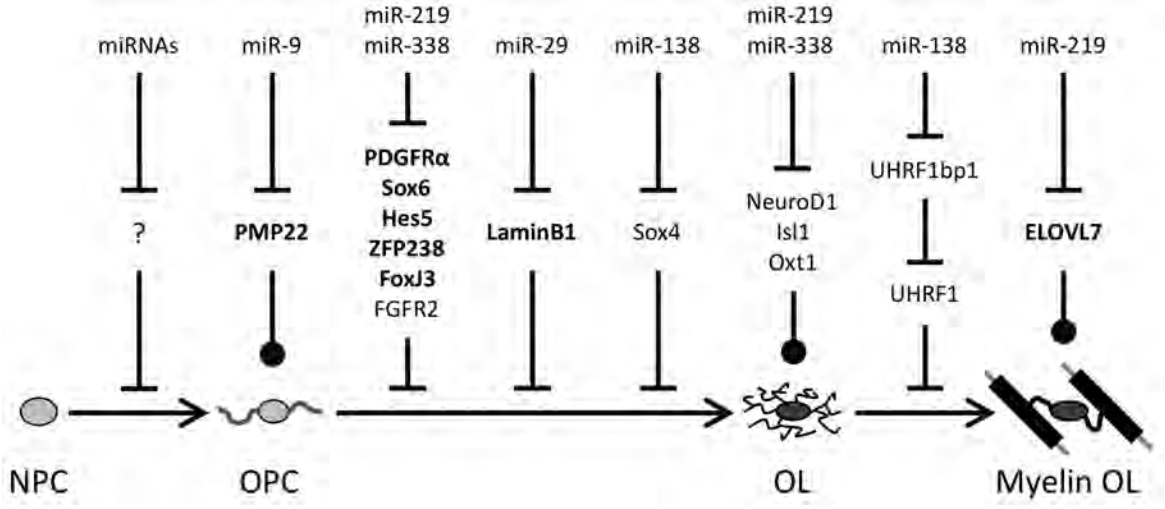

Figure 1. miRNA regulation of CNS myelination. Specific miRNAs involved in regulating various stages of OL differentiation and myelination are shown (or miRNAs in general in the neural precursor to OPC transition). Targets of miRNAs are shown; confirmed targets are in bold and predicted targets in plain text. miR-9 is more highly expressed in OPCs, whereas all other miRNAs shown are more highly expressed in OLs. Inhibition of expression or stage transition is shown by lines with bars; expression that may be detrimental to cell health or function is shown by lines with circles.

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Influences of Individual miRNAs on Oligodendrocyte Biology

Having determined that mature miRNAs are required for normal OL generation and myelin formation, several labs have subsequently investigated the roles of individual miRNAs in promoting functional CNS myelination. These experiments have identified specific miRNAs that promote the formation and maintenance of healthy CNS myelin by three distinct mechanisms:

- The suppression of OPC-expressed genes to promote differentiation;
- The overall suppression of inappropriate non-OL lineage gene expression in OPCs and OLs; and
- The suppression of genes transiently required at high levels during myelin sheath formation.

miRNA promotion of OL differentiation

miR-219

Several labs have noted that miR-219 is the most highly expressed/strongly induced miRNA in differentiating OLs (Lau et al., 2008; Dugas et al., 2010; Zhao et al., 2010) In addition, miR-219 expression appears to be restricted to the vertebrate CNS and to be restricted to mature OLs within the CNS (Wienholds et al., 2005; Dugas et al., 2010), indicating that miR-219 is highly enriched within OLs relative to all other vertebrate tissues. Functionally, miR-219 alone is both necessary and sufficient to promote normal OPC differentiation into OLs *in vitro* and *in vivo*.

miR-219 appears to exert its effects, at least in part, by suppressing the production of several OPC-expressed proteins that normally hinder OL differentiation: PDGFRα, the receptor for the OPC mitogen PDGF, is directly suppressed by miR-219, as are the differentiation-inhibiting transcription factors Sox6, Hes5, ZFP238, and FoxJ3 (Dugas et al., 2010; Zhao et al., 2010). These data indicate a model whereby miR-219 links the initiation of OL differentiation to the suppression of OPC proliferation. By strongly inducing miR-219 at the outset of OL differentiation, the OPC is able to simultaneously suppress the production of several genes that normally maintain the OPC in a proliferative, undifferentiated state, thereby facilitating the rapid state change from proliferating precursor to postmitotic, differentiated OL. Indeed, this model of miRNA function as increasing the gain of a developmental state change has been postulated previously (Reinhart et al., 2000; Bartel, 2009) and, therefore, the results observed for miR-219 may represent one general mode of action of miRNAs in development.

miR-338

miR-338 has also been detected as an miRNA that is strongly induced during OL differentiation and been shown to target the proliferation-promoting genes *Sox6*, *Hes5*, and *ZFP238* (Lau et al., 2008; Dugas et al., 2010; Zhao et al., 2010). However, miR-338 expression appears to be less widespread *in vivo*, as strong expression of miR-338 is detected only in the spinal cord, and significantly weaker expression has been detected in the brain (Dugas et al., 2010; Zhao et al., 2010). In addition, only Zhao et al. (2010) were able to detect a functional role for miR-338 in promoting OL differentiation, whereas Dugas et al. (2010) did not. These differing results may potentially be explained by the fact that miR-338 is also predicted to target FGFR2. Altering miR-338 activity may produce significant effects only on OPCs cultured in the presence of FGF for this reason: FGF is a mitogen for OPCs, but FGF was present only in experiments performed by Zhao et al. but not Dugas et al. In addition, Dugas et al. used only miR-338-5p in experiments, whereas Zhao et al. used both miR-338-5p and miR-338-3p. It is the 3p strand that targets FGFR2 and ZFP238. Therefore, the 3p strand of miR-338 may significantly contribute to the promotion of OL differentiation observed by Zhao et al. Despite these caveats, miR-338 appears to function similarly to miR-219: to increase the rate of OL differentiation by inhibiting the production of OPC-expressed proliferation-promoting genes.

miR-23

miR-23a and miR-23b are both induced ~5× during OL maturation, and overexpression of either can enhance OL differentiation (Lau et al., 2008; Lin and Fu, 2009). miR-23 represses Lamin B1 expression, which is normally downregulated during OL differentiation; overexpression of Lamin B1 inhibits the normal morphological differentiation of OLs, and *Lmnb1* duplication in humans leads to late loss of healthy myelin in autosomal dominant leukodystrophy (Padiath et al., 2006; Lin and Fu, 2009). Therefore, miR-23 appears to also influence OL differentiation by reducing the expression of a gene that inhibits normal OL maturation.

miR-138

miR-138, which is also induced in differentiating OLs, appears to play an interesting role in regulating OL differentiation, which proceeds in a series of distinct temporal stages (Baumann and Pham-Dinh, 2001; Dugas et al., 2006). Whereas miR-219 promotes all stages of OL differentiation, miR-138 specifically promotes the early stages (CNP^+ and MBP^+) of OL differentiation while suppressing the later (MOG^+) stage (Dugas et al., 2010). This

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intermediate MBP+/MOG– stage corresponds to the point at which early differentiating OLs extend processes to contact axons and initiate myelin sheath formation; by the time OLs are MOG+, they have lost the ability to form new myelin sheaths (Watkins et al., 2008). Potentially, miR-138 could play a central role in prolonging this intermediate stage of OL differentiation, which would extend the time frame in which a newly differentiating OL could form the contacts that will produce mature myelin sheaths. How miR-138 accomplishes this remains unclear, but potentially, miR-138 could simultaneously target one set of genes that represses the initiation of OL differentiation and another set of genes that promotes the late stage of differentiation. Candidates include *Sox4*, which pairs with *Sox6* to inhibit early OL differentiation (Stolt et al., 2006), and *UHRF1bp1*, a putative binding partner of *UHRF1*, which itself has been shown to specifically inhibit the late phase of OL differentiation (Dugas et al., 2006).

miRNA suppression of inappropriate gene expression

Often, the expression patterns of genes targeted by an miRNA are inversely correlated with the expression of the targeting miRNA. For example, as miR-219 expression levels rise in differentiating OLs, the expression of several genes targeted by miR-219 falls (Dugas et al., 2010). However, this is not always the case.

miR-9

In one of the earliest studies of miRNA expression in OL-lineage cells, miR-9 was one of two OPC-enriched miRNAs identified whose expression positively correlated with its predicted targets (Lau et al., 2008). This correlated expression pattern may indicate miR-9's role in repressing inappropriate gene expression: miR-9 may be expressed to silence the "leaky" expression of genes that should not be produced in OL-lineage cells and would therefore be required at highest levels when its targeted genes are also being most highly expressed. In fact, this appears to be the case for at least one gene miR-9 directly targets: *PMP-22*. PMP-22 is normally produced only in PNS-myelinating Schwann cells, yet *PMP-22* mRNA expression is observed in OPCs (Baumann and Pham-Dinh, 2001; Dugas et al., 2006; Cahoy et al., 2008). Lau and colleagues (2008) demonstrated that miR-9 directly prevents the inappropriate production of PMP-22 protein in OL-lineage cells. These data illustrate that miRNAs play an additional role as "guardians of the transcriptome" by preventing inappropriately expressed mRNAs from being translated into functional proteins that could detrimentally affect the health of the cell.

miR-219 and miR-338

In addition to the role miR-219 and miR-338 play in promoting OL differentiation, Zhao et al. (2010) point out that these miRNAs may also play a role in inhibiting the production of neurogenic factors in OLs, such as NeuroD1, Isl1, and Otx1—all targets of one or both of these miRNAs. The fact that miR-219 and miR-338 could be involved both in extinguishing OPC gene expression and blocking inappropriate neurogenic gene expression illustrates the fact that miRNAs, by virtue of the wide variety of genes they are capable of targeting, can simultaneously influence distinct aspects of a cell's gene expression program.

miRNA suppression of genes transiently required during differentiation

miR-219

miR-219, in addition to its role in promoting OL differentiation and putative role in preventing inappropriate neurogenic gene expression, contributes to the regulation of gene expression in fully mature OLs. Robust OL expression of miR-219 has been consistently detected in adult (P50-60) mice, and the expression of miR-219 is lost when Dicer1 function is specifically ablated in mature OLs in mice containing a tamoxifen-inducible *PLP-CreER*^{T} gene (Shin et al., 2009; Dugas et al., 2010). Interestingly, disruption of OL-expressed Dicer1 at P14-18 leads to a strong reduction in miR-219 levels in mature OLs by P30, but at this age, mutant mice look normal and begin to show functional deficits only by P60-90, with reduced CNS myelin observed at P180. So why the delay, and what is the reason for the eventual loss of healthy CNS myelin? In these mice, Dicer1 function is disrupted only in mature OLs, so OL specification and differentiation should not be adversely affected. Instead, these results indicate a role for mature miRNAs in maintaining healthy myelin, with the caveat that miRNAs in mature OLs are more acutely required after myelin has been fully formed by P45-60 (Baumann and Pham-Dinh, 2001) as opposed to during the initial generation of myelin sheaths.

This dichotomy may be explained by the fact that a prominent target of miR-219 is *ELOVL7* (Shin et al., 2009). ELOVL7 is an enzyme expressed at high levels in OLs that is involved in the production of very-long-chain fatty acids (VLCFAs) (Cahoy et al., 2008; Tamura et al., 2009). VLCFAs are incorporated into proteolipid protein (PLP) as an integral component of the fatty myelin sheath, but overproduction of VLCFAs can lead to demyelinating diseases such as X-linked adrenoleukodystrophy (Dubois-Dalcq et al., 1999). Potentially, high levels

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of ELOVL7 activity could be required during active myelin sheath formation, when large amounts of lipid-rich membranes are being produced, but this production would need to be tempered once full axonal myelination is completed. miR-219 may serve this role in mature OLs by reducing the amount of functional ELOVL7 produced from the highly transcribed *ELOVL7* locus.

In general, these data indicate that another role for miRNAs in OLs (and likely in other cell types) may be to moderate the expression of proteins that are required at high levels for the transformation from immature to fully differentiated phenotype but whose continued high-level expression in stable, mature cells may be detrimental. As a consequence of the continued expression of transcription factors that specify the mature state of the cell, expression of these genes would persist but could be regulated by miRNAs' suppression of targeted genes.

miRNA in glioma

The role of miRNAs in promoting OL differentiation by inhibiting OPC-expressed proliferation-promoting genes may also indicate that miRNA misregulation could contribute to glioma proliferation; if OL-expressed miRNAs inhibit proliferation, then loss of these miRNAs would create a permissive environment for tumorigenesis. In fact, in analyzed medulloblastoma samples, OL-expressed miR-219, miR-138, and miR-192 are all downregulated relative to normal control tissue expression (Ferretti et al., 2009). Cumulatively, these data indicate that reintroducing OL-enriched miRNAs into active CNS tumors, especially those expressing characteristics of the OPC-OL lineage, could prove efficacious for blocking tumor progression and/or driving tumor regression.

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