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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