

MINIREVIEW

Regulation of the Mammalian Nervous System by MicroRNAs

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ABSTRACT

The mammalian nervous system exerts essential control on many physiological processes in the organism and is itself controlled extensively by a variety of genetic regulatory mechanisms. MicroRNAs, a class of small, noncoding RNAs, are critical contributors to the regulation of gene expression in the nervous system. Emerging evidence indicates that microRNAs

regulate both the development and function of the nervous system. Deficiency in microRNA function has also been implicated in a number of neurological disorders. Understanding the roles of microRNAs will provide new insights into the complexity and operation of the nervous system.

The nervous system undergoes extensive changes in patterning, remodeling, and cell specification during development. In mature mammals, it consists of networks of cells that reach every organ and part of the body to conduct impulses back and forth to control essential physiological responses to internal and external stimuli in a timely fashion. To accomplish its tasks, the nervous system uses a large number of cells with different properties to form exceedingly complex structures and depends on an array of elaborate gene regulatory mechanisms for its development and function. microRNAs (miRNAs) have been added as the newest key players in the regulation of the nervous system. miRNAs are a class of abundant, approximately 22-nucleotide-long RNAs endogenously expressed in a wide range of organisms and in every cell type of the organisms. By regulating the expression of a large number of protein-encoding genes, miRNAs control a variety of important biological processes (Ambros, 2004). This review summarizes our current understanding of the roles of miRNAs in the mammalian nervous system.

miRNAs: the Biogenesis and the Mechanisms of Action. An miRNA may be located within an intron or exon of a host gene or constitute an independent transcription unit

(Rodriguez et al., 2004). It is transcribed initially as part of a much longer primary transcript, usually by RNA polymerase II (Cullen, 2004). In mammals, the transcript is cleaved by an RNase called Drosha, along with its regulatory subunit DGCR8, to liberate an approximately 65-nucleotide hairpin precursor in the nucleus. A small number of precursors can also be generated in a Drosha-independent manner (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). The precursor is then exported to the cytoplasm by Exportin5 and its Ran cofactor bound to GTP. Once in the cytoplasm, the precursor is further processed by another RNase, Dicer, to produce an approximately 22-base pair RNA duplex intermediate. The binding of an Argonaute protein to the duplex and the ensuing structural rearrangements result in the retention of the mature, single-stranded miRNA in the Argonaute:miRNA complex. Like mRNA expression, miRNA expression can be regulated transcriptionally and post-transcriptionally, and some of the examples will be discussed later.

The Argonaute:miRNA complex mediates the direct biological effects of the miRNA via RNA interference and related mechanisms (He and Hannon, 2004). Numerous proteins have been reported to interact with the Argonaute protein, although their subsequent functions have not been firmly established. The miRNA moiety clearly provides specificity to the RNA silencing process by binding to its target sequence, usually located in the 3'-untranslated regions of an animal mRNA. Complementarity between the 5'-end of the miRNA, the so-called seed region, and the target mRNA seems to be disproportionately critical for the binding specificity, whereas the

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ABBREVIATIONS: miRNA, microRNA; Limk1, lim-domain containing protein kinase 1.

3'-end of the miRNA makes less contribution to target recognition (Lewis et al., 2005). Because an animal miRNA is almost never perfectly matched to its targets, and partial complementarities are indeed sufficient for miRNA function, an miRNA may regulate the expression of hundreds of genes; on the other hand, an mRNA may contain multiple miRNA-targeting sites (Lewis et al., 2005; Xie et al., 2005; Miranda et al., 2006). The interaction between an miRNA and its target mRNA principally leads to decreased production of the target gene product (i.e., protein), although the detailed mechanism remains elusive (Filipowicz et al., 2008). The Argonaute protein probably interacts with the translation machinery to inhibit protein synthesis, which might occur at various stages (e.g., the initiation and elongation steps) during translation, perhaps depending on the nature of the miRNA and target transcript. mRNAs that are prevented from translation often show reduced accumulation as well. Additional modes of action have also been ascribed to miRNAs. For example, miRNAs may repress gene expression in cycling cultured cells but enhance gene expression in arrested cells (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). Although the latter possibility has significant implications for postmitotic neurons, research efforts so far have been focused on understanding miRNA-mediated gene repression in the nervous systems.

There are approximately 600 human miRNA genes in the current miRNA database, encoding approximately 1000 potential miRNAs (Griffiths-Jones et al., 2008). Many of them are evolutionarily conserved in mammals, some even in worms and flies. miRNA genes are named in the order of their discovery, such as miR-1, miR-2, etc., taken into consideration of species conservation, with the exception of *lin-4* and *let-7*, which are the first two miRNAs ever identified. miRNA discovery has been greatly facilitated by massive sequencing efforts and by computer program prediction followed by confirmation with sensitive polymerase chain reaction methods. These approaches, however, have caveats. A small number of the miRNAs are probably misannotated and instead represent degradation products of unrelated transcripts (Berezikov et al., 2006a). Moreover, because an miRNA acts by binding to its target mRNAs, potentially numbered in the hundreds, the function of a miRNA critically depends on its mass. The copy number of the most abundant miRNAs can well exceed 10,000 per cell or neuron (Lim et al., 2003; Kye et al., 2007), but it is possible that certain database miRNAs are expressed at too low a level to be effective against most of its otherwise potential targets. On the other hand, even if an miRNA is found rarely in a total tissue sample, it can still be functional if it is highly restricted to a subpopulation of cells of a particular cell type or developmental stage, which may be relevant to the situation in the nervous system.

miRNA Expression in the Nervous System. Like other tissues and cells, the nervous system and neural cell lines also express miRNAs, some of which are enriched or unique in the tissue and neural cells (e.g., miR-9, miR-124, miR-125, miR-128, and miR-129) (Lagos-Quintana et al., 2002; Dostie et al., 2003; Babak et al., 2004; Barad et al., 2004; Kim et al., 2004; Liu et al., 2004; Nelson et al., 2004; Sempere et al., 2004; Baskerville and Bartel, 2005; Berezikov et al., 2006b; Hohjoh and Fukushima, 2007a; Landgraf et al., 2007; Bak et al., 2008). The number of miRNA genes found to be expressed in the nervous system seems to be larger than that in many

other organs, perhaps partly reflecting the fact that the nervous system contains many types and subtypes of cells. Toward understanding the complexity of miRNA expression, these studies have further revealed that anatomically distinct areas of the adult central nervous system (e.g., cerebellum, hypothalamus, and hippocampus) express similar miRNAs, but relative miRNA levels can vary significantly in different regions.

miRNA expression during neuronal differentiation and neurodevelopment has also been investigated. When treated with all-*trans*-retinoic acid, embryonal carcinoma cells will terminally differentiate into neuron-like cells. Accompanied with the morphological changes, expression of miRNAs such as miR-9, miR-124, and miR-125 is significantly induced over time, suggesting that these miRNAs may play a role in differentiation or cell fate determination, in addition to their potential functions in adults (Sempere et al., 2004; Smirnova et al., 2005; Hohjoh and Fukushima, 2007b). Many miRNAs that are not specific to the nervous system are also affected. For example, the *let-7* family of miRNAs is prominently up-regulated, which probably have a more general influence on the process of differentiation and development. Similar and profound changes in miRNA expression are observed when embryonic stem cells undergo neurogenesis and gliogenesis (Smirnova et al., 2005; Krichevsky et al., 2006). Furthermore, miR-124 and miR-128 are shown to be preferentially expressed in neurons, whereas miR-23, miR-26, and miR-29 are restricted to or enriched in astrocytes (Smirnova et al., 2005). miRNA expression profile in nervous system development in mammals has also been examined, and again, a temporally regulated wave of miRNA expression is observed (Krichevsky et al., 2003; Miska et al., 2004; Smirnova et al., 2005; Wheeler et al., 2006; Dogini et al., 2008). All of these results suggest that miRNA expression profile can serve as a marker of neuronal development and that specific miRNAs may contribute to the developmental process.

miRNAs have been isolated from polysomes in cultured neurons, consistent with miRNA's role in controlling translation (Kim et al., 2004; Nelson et al., 2004). A strategic facet of gene regulation in neural cells is that many mRNAs are concentrated near specific structures to ensure local, activity-regulated protein synthesis. It is conceivable that some miRNAs also follow such subcellular distribution patterns. Indeed, selective enrichment or depletion of miRNAs in the dendrites has been reported (Schratt et al., 2006; Kye et al., 2007). These results suggest that miRNAs, like sequence-specific mRNA-binding proteins, could regulate gene expression locally to affect synaptic plasticity in neural cells.

miRNA Function: Lessons from the Studies of the Global Loss of miRNAs. Conditional knockouts of *Dicer*, the gene required for miRNA biogenesis, have been used extensively to examine the collective roles of miRNAs in specific tissues and cell types in mice. Loss of *Dicer* in mature Purkinje cells is followed by rapid dissemination of miRNAs without immediate impact on cell physiology or function (Schaefer et al., 2007). Nonetheless, cell death eventually occurs, leading to progressive cerebellar degeneration and development of ataxia, which mirrors neurodegenerative disorders in humans. *Dicer* ablation in postmitotic midbrain dopaminergic neurons also leads to a progressive loss of the neurons *in vitro* and *in vivo*, and mutant mice have markedly reduced locomotion, reminiscence of patients with Parkin-

son's disease (Kim et al., 2007). Homozygous knockout of Dicer, starting in embryonic day 15.5, in the cortex and hippocampus of mice results in changes in dendrite morphology, apoptosis, microcephaly, ataxia, and death by 3 weeks after birth (Davis et al., 2008). Mice with Dicer loss in striatal dopaminergic neurons also display behavioral and neuro-anatomical phenotypes, although, unlike neurons targeted in the other studies, the affected neurons survive over the lifespan of the animals, which is approximately 10 weeks (Cuellar et al., 2008). Dicer is further required for olfactory differentiation in the embryo, the maintenance of olfactory progenitors, and the differentiation of olfactory precursors, whereas it is dispensable for the proper function of the mature neurons in mice (Choi et al., 2008). An underlying cause of these phenotypes may be that miRNA depletion leads to a very gradual loss of important proteins and/or accumulation of certain proteins to a level that is ultimately toxic to cells. Uncertainty remains as to whether some of the observed phenotypes result from the loss of miRNA-independent functions of Dicer, because Dicer processes other small RNAs, such as small interfering RNAs, as well. Haploinsufficiency of DGCR8, another gene involved in miRNA processing, results in reduced miRNA expression and neuronal and behavioral deficits in mice as well (Stark et al., 2008). Overall, a very strong case can be made for the important functions of miRNAs in neuronal differentiation and survival, which is consistent with the ubiquitous expression of miRNAs and their functions in other tissues.

miRNA Function: Lessons from the Studies of Individual miRNAs. The functions of individual miRNAs in developing neurons have been investigated. In the same study that demonstrated the combined roles of miRNAs in maintaining midbrain dopaminergic neurons (Kim et al., 2007), **miR-133b** was found to repress the differentiation of these neurons from embryonic stem cells and midbrain cultures. The authors identified a target of miR-133b as the transcription factor *Pitx3*, which normally activates gene expression in dopaminergic neurons. Choi et al. (2008) showed that **miR-200** is essential for the differentiation of olfactory progenitor cells and that its function may depend on its ability to target the Notch and transforming growth factor- β signaling pathways and *Foxg1*. Another perhaps best-studied example is **miR-124**, an abundant and signature miRNA in neurons. miR-124 expression is low in embryonic stem cells and neuronal precursor cells but elevates dramatically in neurons. Early overexpression of miR-124 along with another abundant miRNA, miR-9, shifts precursor differentiation to neurons, suggesting that miR-124 and miR-9 stimulate neuronal differentiation (Krichevsky et al., 2006). In a separate study, miR-124 overexpression promotes whereas inhibition of miR-124 function delays neurite outgrowth (Yu et al., 2008). miR-124 may confer neuronal properties to cells, because miR-124 overexpression in HeLa cells down-regulates many genes whose expression is absent in neurons (Lim et al., 2005), whereas blocking miR-124 activity in mature neurons increases the levels of non-neuronal mRNAs (Conaco et al., 2006). miR-124 executes its functions by at least three mechanisms. First, it inhibits the expression of the small C-terminal domain phosphatase 1, a component of the RE1-silencing transcription repressor (Visvanathan et al., 2007). In non-neuronal tissues, the RE1-silencing transcription repressor shuts down the transcription of many

neuronal genes, including miR-124 (Conaco et al., 2006), which is an emerging example of critical transcription factors regulating the expression of both mRNAs and miRNAs. As a result of increased miR-124 in neurons, the transcription of many neuronal-specific genes is induced. Second, miR-124 blocks the expression of polypyrimidine tract-binding protein 1, a global repressor of neuron-specific, alternative exon inclusion in non-neuronal cells (Makeyev et al., 2007). Thus, miR-124 manages two master regulators to influence the expression of a broad spectrum of genes. Third, miR-124 directly targets many genes involved in cytoskeletal regulation, which may explain its function in promoting neurite outgrowth (Yu et al., 2008). miR-124 probably has many other direct targets as well.

In mature neurons, miRNA-regulated local protein synthesis at synapses is an attractive model for establishing synaptic plasticity. In rat hippocampal neurons **miR-134** is concentrated in the synaptodendritic compartment (Schratt et al., 2006). Overexpression of miR-134 significantly decreases the volume of dendritic spines, which approximates synaptic strength, whereas inhibiting miR-134 function increases spine volume. At the dendrites, miR-134 prevents the translation of the lim-domain containing protein kinase 1 (*Limk1*), a regulator of actin filament dynamics. Overexpression of *Limk1* counteracts the effects of miR-134 on spine morphology, indicating that inhibition of *Limk1* expression is a major pathway through which miR-134 restrains the size of dendritic spines. The functional interaction between *Limk1* and miR-134 can be regulated by neuronal activities, because it is relieved by the brain-derived neurotrophic factor released upon synaptic stimulation through yet-to-be-determined mechanisms. The implication is that if the association of an miRNA, like that of RNA-specific binding proteins, with a target mRNA or mRNAs is controlled by a stimulus, then the stimulus can modulate the interaction between the miRNA and mRNA(s) to regulate gene expression quickly and coordinately. Although miR-134 is so far the only mammalian miRNA shown to have a localized function in neurons, the finding that proteins involved in miRNA biogenesis and function are present in postsynaptic densities, axons, and growth cones suggests that the specific functions of additional miRNAs may be identified at such locations (Lugli et al., 2005; Hengst and Jaffrey, 2007). Hinting at a role for miRNAs in controlling neurotransmitter release, it has been reported that **miR-130a** and **miR-206** inhibit the synthesis of the neurotransmitter substance P in human mesenchymal stem cell-derived neuronal cells, whereas interleukin-1 α reduces the expression of the miRNAs, thereby relieving the inhibition (Greco and Rameshwar, 2007).

The expression and function of neural miRNAs is influenced by external cues, including pharmacological agents. In a fetal mouse cerebral cortex-derived neurosphere culture model to study how ethanol affects fetal brain development, a high dose of ethanol is shown to suppress the expression of miR-21, miR-335, miR-9, and miR-153, but a lower dose of ethanol induces miR-335 (Sathyan et al., 2007). Reactive oxygen species alter miRNA expression in human brain cell cultures (Lukiw and Pogue, 2007), a situation that may bear relevance to Alzheimer's disease (Lukiw, 2007). As an example of psychotherapeutic drugs targeting miRNAs, lithium, and valproate, two important mood stabilizers, affect the long-term expression of *let-7b*, *let-7c*, miR-128a, miR-24a,

miR-30c, miR-34a, miR-221, and miR-144 in rat hippocampus (Zhou et al., 2008). Functions of these miRNAs need to be better defined. The miRNAs might partly mediate the effects of ethanol, reactive oxygen species, or mood stabilizers on gene expression, and/or they might signify the adaptive changes in brain cells. From the changed miRNAs, one can deduce and test the expression changes in their target genes to shed light on the mechanisms of action by various agents and treatments. In one such study, long-term hyperosmolar stimulation is shown to increase **miR-7b** levels in the hypothalamus, and an miR-7b target is identified as Fos, a critical transcription factor that mediates responses to many neuropharmacological agents (Lee et al., 2006). The transcription of **miR-132** is positively controlled by the cAMP response element binding protein, which, like Fos, responds to a wide range of stimuli and neural activities (Vo et al., 2005; Wayman et al., 2008). miR-132 down-regulates p250GAP, a member of the Rac/Rho family of GTPase-activating proteins that restricts neurite outgrowth. Activity-driven cAMP response element binding protein-dependent production of miR-132 results in p250GAP inhibition and neurite outgrowth, thereby contributing to dendritic plasticity. A second target of miR-132 is the methyl CpG-binding protein 2, a general transcription repressor (Klein et al., 2007). In addition, miR-132 and another brain-specific miRNA, miR-129, are controlled by light and the circadian clock and in turn modulate the circadian-timing process in the suprachiasmatic nucleus *in vivo* (Cheng et al., 2007).

From the fast-growing body of evidence, it is clear that miRNAs regulate the expression of genes involved in a diverse range of processes to affect many steps and aspects of the maturation and operation of the mammalian nervous system. Future studies will elucidate how miRNAs act, in concert with transcription factors, mRNA binding proteins, and other regulatory proteins, to fine-tune gene expression in response to internal and external stimuli temporally and spatially.

miRNA Association with Neurological Diseases in Humans. Aberrant miRNA expression and function has been implicated in cancer and other disorders in the nervous system. miRNAs are differentially expressed in glioblastoma and neuroblastoma (Chan et al., 2005; Ciafre et al., 2005; Laneve et al., 2007; Lukiw et al., 2009; Silber et al., 2008). For example, glioblastoma has increased levels of miR-21, miR-221, and miR-222 but decreased levels of miR-7, miR-124, and miR-137. miR-21 is a suspected oncogene frequently overexpressed in cancers. Potential targets of miR-221 and miR-222 include p27 and p57, inhibitors of cell cycle progression (Gillies and Lorimer, 2007; Medina et al., 2008), whereas decreased miR-7 may up-regulate the expression of epidermal growth factor receptor and the Akt pathway (Kefas et al., 2008) and Fos (Lee et al., 2006).

Numerous Dicer knockout studies have revealed mouse phenotypes similar to those exhibited in human neurodegenerative diseases (see above), suggesting that the loss of global and/or specific miRNAs may contribute to the diseases. In humans, a single-nucleotide polymorphism in the **miR-189** binding site in the 3'-untranslated region of the mRNA coding for a strong candidate gene for Tourette's syndrome, SLIT and Trk-like 1, has been identified (Abelson et al., 2005). The nucleotide change enhances miRNA-mediated gene repression, according to a reporter assay. **miR-133b** expression is

deficient in the midbrain of patients with Parkinson's disease, although the causative relationship between miR-133b loss and Parkinson's disease awaits determination (Kim et al., 2007). A number of miRNAs are found differentially expressed in the prefrontal cortex of patients with schizophrenia (Perkins et al., 2007) or in the brains of patients with Alzheimer's disease (Lukiw, 2007; Lukiw et al., 2008). For example, miR-146a is elevated in the brain cells of patients with Alzheimer's disease, whereas the expression of its putative target, complement factor H, is depressed. Transcription of miR-146a is stimulated by nuclear factor- κ B (Taganov et al., 2006; Lukiw et al., 2008), which is consistent with the involvement of inflammatory and other stress responses in the pathogenesis of Alzheimer's disease. Alzheimer's disease is further correlated with the loss of brain **miR-29** and **miR-107**, which normally suppress β -secretase expression (Hebert et al., 2008; Wang et al., 2008). Finally, altered expression of miRNAs, including reduced **miR-132**, is reported in patients with Huntington's disease (Johnson et al., 2008). Once a correlation is established between miRNA expression and neurological disorders, a daunting task is to elucidate the contribution of miRNAs to these various diseases.

Therapeutic Intervention Based on Our Knowledge of miRNAs. Because of the differential expression of miRNAs in various diseases, it is tempting to overexpress miRNAs or to inhibit miRNA function to treat such ailments. Although any results are still preliminary, inhibition of **miR-21** function is shown to induce apoptosis in glioblastoma cells and sensitizes the cells to cytotoxic tumor therapy in mice (Chan et al., 2005; Corsten et al., 2007). Overexpression of **miR-221** and **miR-222** in glioblastoma cells promotes premature cell cycle entry, leading to cell death (Medina et al., 2008). Likewise, **miR-7** overexpression decreases viability and invasiveness of primary glioblastoma lines *in vitro* (Kefas et al., 2008). These studies demonstrate that differential miRNA expression has functional consequences and that the miRNAs may serve as targets for drug intervention. For example, cholesterol-conjugated miRNAs or their inhibitors, or their viral expression vectors, can be introduced by targeted injection into the brain to alter miRNA function. On the other hand, drugs that regulate miRNA expression can be developed, or once the downstream effectors of the miRNAs are revealed, they will become drug targets as well.

Artificial miRNAs or short hairpin RNAs have also been designed and used to repress gene expression via RNA interference in disease models. In such cases, the miRNAs function as small interfering RNAs to target viral genes or endogenous genes known to cause diseases. In one study, a single intracranial administration of lentiviral-encoded short hairpin RNAs protects mice from lethal encephalitis induced by the Japanese encephalitis virus (Kumar et al., 2006). In another study, intracerebellar injection of recombinant adeno-associated viruses expressing short hairpin RNAs against mutant ataxin-1, the protein responsible for the disease spinocerebellar ataxia type 1, improved motor coordination, restored cerebellar morphology, and lessened nuclear ataxin-1 inclusions in a murine disease model (Xia et al., 2004). A third study targets Huntington's disease (McBride et al., 2008), which is caused by a dominantly mutant huntingtin protein. Artificial miRNAs against the protein are encoded by recombinant adeno-associated viruses and delivered via injection into the striatum of mice expressing the

mutant human huntingtin protein. The miRNAs are able to reduce mutant huntingtin expression without causing apparent toxicity in mouse brain.

Future Perspective. There is no doubt that the collection of validated miRNA targets and functions is going to expand at a brisk pace in the near future. To further our understanding of the complex roles of miRNAs in regulating the nervous system, addressing a number of the following issues will be highly beneficial. First, is it possible to refine the resolution of miRNA expression to accommodate the many different types and subtypes of cells in the developing and mature nervous system? In addition, is the subcellular localization of miRNAs dynamic, and is miRNA function spatially regulated in a neural cell? Second, a systems or global view should be adopted to evaluate how changes in miRNA expression lead to changes in protein expression and, ultimately, changes in phenotypes. An miRNA probably has many targets. Although published reports have examined, for every miRNA, typically only one of its targets whose activity is consistent with the overall function of the miRNA, it is highly probable that a miRNA can regulate genes that both positively and negatively control any particular process in vivo. The actions of miRNAs are also integrated with the actions of other regulatory molecules (e.g., transcription factors). Third, genetics approaches (e.g., conditional knockout of individual miRNAs) will give us more definitive answers on the functions of miRNAs in the mammalian nervous system. Genetics analyses in worms, flies, and zebrafish have greatly advanced our knowledge of miRNAs and indeed have foretold or corroborated many findings in the mammalian system. Finally, the causal links between miRNAs and neurological disorders need to be established, and such information should be used to devise new therapeutic strategies.

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