MicroRNAs: Not “Fine-Tuners” but Key Regulators of Neuronal Development and Function

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MicroRNAs (miRNAs) are a class of short non-coding RNAs that operate as prominent post-transcriptional regulators of eukaryotic gene expression. miRNAs are abundantly expressed in the brain of most animals and exert diverse roles. The anatomical and functional complexity of the brain requires the precise coordination of multilayered gene regulatory networks. The flexibility, speed, and reversibility of miRNA function provide precise temporal and spatial gene regulatory capabilities that are crucial for the correct functioning of the brain. Studies have shown that the underlying molecular mechanisms controlled by miRNAs in the nervous systems of invertebrate and vertebrate models are remarkably conserved in humans. We endeavor to provide insight into the roles of miRNAs in the nervous systems of these model organisms and discuss how such information may be used to inform regarding diseases of the human brain.

Keywords: microRNA, brain, neurogenesis, axon guidance

INTRODUCTION

MicroRNAs (miRNAs) are non-coding RNA molecules with a length of approximately 22 nucleotides, which act as post-transcriptional regulators of gene expression (1–4). Discovered just over two decades ago, miRNAs have been found to be abundant in most organisms and critically important for post-transcriptional control of mRNAs by regulating a predicted 60% of protein-coding genes (5). Prior to the discovery of the miRNA pathway, the lin-14 gene in Caenorhabditis elegans was shown to be regulated by a 22-nucleotide partially complementary strand of RNA called lin-4 (6, 7). However, a mechanistic understanding of this process remained unclear until the let-7 gene was shown to encode a complementary sequence of lin-41 to regulate developmental timing (8). This led to a paradigm shift in how mRNA regulation was viewed, and further investigation demonstrated that the miRNA pathway was evolutionarily conserved in most eukaryotes (9). Since then miRNAs have been shown to be required for key biological processes, such as cell fate, differentiation, apoptosis, and tumor suppression (10–13).

The process of miRNA biogenesis in animals can be briefly simplified into three fundamental steps (Figure 1) [for detailed review, see Ref. (14)]. First, double-stranded primary miRNA (pri-miRNA) short hairpin structures are transcribed by RNA polymerase II. Secondly, a nuclear-localized RNA endonuclease III, Drosha, defines one end of the pri-miRNA duplex and cleaves double-stranded RNA (dsRNA) transcripts into approximately 70 nt stem loops called precursor miRNAs (pre-miRNAs) (15). These pre-miRNAs are exported to the cytoplasm by Exportin-5 (XPO5) (16) where the Dicer enzyme cleaves pre-miRNA sequences into 21–23 nt mature miRNA double-stranded duplexes (17). Such miRNA duplexes load into a pre-RISC (pre-miRNA-induced silencing complex) which is a
complex of Argonaute (AGO) and other proteins (18). Within the pre-miRISC, the “passenger” strand is removed leaving just the “guide” strand in the mature miRISC. The guide strand is normally the strand with a more thermodynamically unstable 5' end or “seed” region of miRNAs (3). The released passenger strand is either degraded or loaded into a different miRISC complex to regulate a different group of target transcripts. These sequences are either repressed by the RISC complex or degraded in P-bodies.

As mentioned above, the nature of miRNA targeting through imperfect complementarity means that single miRNAs have the potential to regulate the expression of hundreds of genes (3). In addition, certain genes have multiple miRNA-binding sites in their 3' UTRs and, therefore, multiple miRNA families potentially control their expression (3). 3' UTR length is often a determining factor as to its propensity to miRNA regulation (3). Such complex relationships between miRNAs and their targets enable exquisite control of gene regulatory networks. A better understanding of miRNA function in the control of such gene regulatory networks has been accelerated by the use of simple model organisms, such as C. elegans and Drosophila. Studies using these models are aided by their genetic amenability, short lifespans, and compact genomes. However, there are multiple mammalian-specific miRNAs for which the use of higher eukaryotes is required to study their biology.

FUNCTIONS OF miRNAs IN THE NERVOUS SYSTEM

The human brain contains approximately 86 billion neurons and trillions of synaptic connections (23). This complex organ is an integration center where environmental information is processed and used to make an appropriate action or decision. To effect brain function as a whole, neurons are organized into circuits which communicate with each other through rapidly acting synaptic connections and slower acting neuropeptide release. An inability to regulate these molecular communication processes is causative in developmental disorders, such as autism and schizophrenia, in addition to age-related decline of brain function (24, 25). Therefore, using model organisms to dissect these mechanisms at a molecular, anatomical and functional level will provide a greater understanding of neuronal-based disease.

The ability of the nervous system to adapt to different environmental conditions and stimuli requires a well-conserved and flexible repertoire of molecular mechanisms. miRNAs offer genetic networks’ additional layers of regulatory control and are abundantly expressed in all human tissues, including the brain (26). In addition to this, many miRNAs display specific temporal and spatial patterns of expression (27). Due to the high degree of complexity of the human brain, in addition to ethical concerns, deep mechanistic understanding of how miRNAs influence neurodevelopmental and functional processes has come from model organisms. This review aims to provide examples that reveal the important roles of miRNAs in the development and function of the nervous system (Figure 2). We focus on the crucial role model organism research has played in this area to provide insight into the functions of miRNAs.

miRNA REGULATION OF THE NERVOUS SYSTEM IN INVERTEBRATES

Caenorhabditis elegans

The initial discovery of miRNAs was made in the nematode C. elegans and, since that time, many fundamental studies implicating miRNAs in gene regulatory networks significant to neurodevelopment have been achieved using this model organism research has played in this area to provide insight into the functions of miRNAs.
miRNAs are involved in the multiple stages of neuronal development in invertebrates and vertebrates. Listed here are the miRNAs we cover in this review that regulate single or multiple stages of neuronal development.

- cel, Caenorhabditis elegans
- dme, Drosophila melanogaster
- dre, Danio rerio
- hsa, Homo sapiens
- mmu, Mus musculus
- xtr, Xenopus tropicalis

**Figure 2: Roles of miRNAs in different stages of neuronal development.** miRNAs are involved in the multiple stages of neuronal development in invertebrates and vertebrates. Listed here are the miRNAs we cover in this review that regulate single or multiple stages of neuronal development. cel, Caenorhabditis elegans; dme, Drosophila melanogaster; dre, Danio rerio; hsa, Homo sapiens; mmu, Mus musculus; xtr, Xenopus tropicalis.

C. elegans has proven demonstrative as many miRNAs are highly conserved throughout evolution, along with other well-known advantages of the model organism including its well-defined neuronal lineage map, neuroanatomy, and neural networks (31–35).

MicroRNAs have been shown to participate in instructing cell fate decisions made during the development of the nervous system of C. elegans. For example, the ASE neurons are a pair of morphologically similar, asymmetric gustatory sensory neurons, which have different roles: ASEL senses sodium, while ASER senses chloride (36). The neuron pair is derived from different lineages, which diverge at the four-cell stage of embryogenesis. A complex gene regulatory pathway, in which the miRNA lsy-6 plays a central role, essentially achieves the specification of this pair of neurons during development. The Nkx homeobox transcription factor COG-1, which induces ASER fate, is inhibited by lsy-6 in ASEL (37–39). It was subsequently shown that lsy-6 expression itself is controlled by a complex mechanism involving two regulatory elements, firstly where transcription factors TBX-37/38 "prime" the lsy-6 locus for expression by altering chromatin to an open state. Expression is then "boosted" by the transcription factor CHE-1, and lsy-6 induces gene expression changes associated with asymmetrical generation of the ASEL and ASER neurons (40). This "priming" and "boosting" of lsy-6 begins several cell divisions prior to the specification of ASEL neuron and is the first asymmetrically expressed gene in the ASEL and ASER neurons. This mechanism resulting in bilateral symmetry in the nervous system may provide important insights into how symmetry is established in the mammalian brain.

mir-71 is another miRNA involved in neuron specification in C. elegans. mir-71 controls cellular responses to calcium to specify asymmetry in function of the morphologically symmetrical AWCON and AWCOFF olfactory neurons (41). mir-71 controls this signaling pathway as it is expressed at a higher level in one neuron of the pair, which negatively regulates the calcium adaptor protein TIR-1/Sarm1 downstream of nys-4/claudin and nys-5/innexin, to specify the AWCON neuron (41). Interestingly, for this pair of neurons, cell fate is not rigid. That is, whether the left or right neuron is specified AWCON is not fixed, and if the AWCON is lost, AWCOFF can convert to AWCON (41).

Aside from neuronal specification, evidence from C. elegans indicates that miRNAs are involved in gene regulation to control neuron migration and differentiation. It was demonstrated that a regulatory pathway essential for normal neuron migration and axon guidance involves mir-79 (an ortholog of mammalian miR-9) (42). In C. elegans, loss of mir-79 from epidermal cells caused an increase in expression of sqv-5 (a chondroitin synthase) and SQV-7 (a UDP-sugar transporter), both required for the biosynthesis of glycosaminoglycan (GAG) chains that are attached to proteoglycans (43). Defective regulation of SQV-5 and SQV-7 in the epidermis caused striking defects in the migration of hermaphrodite-specific neurons (HSNs). RNAi knockdown of sqv-5 and sqv-7 in mir-79 deletion mutant animals restored the incidence of HSN defects to background levels. Additional
work showed that this mechanism is required to regulate the addition of heparan sulfate chains on a glypican called LON-2. This work, therefore, defined a pathway through which mir-79 expression in epidermal cells non-cell autonomously controls HSN migration (42).

Netrin-mediated axon guidance is also influenced by miRNA expression in C. elegans to ensure the correct timing of axon termination. Cell autonomous expression of lin-4 (homologous with mir-125a/b) targets the transcription factor lin-14 at completion of extension of the anterior ventral microtubule (AVM) axon, to inhibit netrin-mediated axon attraction (44). Such temporal loss of responsiveness is due to decreased expression of the netrin receptor UNC-40 through lin-4-mediated reduction of LIN-14. The cell-autonomous regulation of lin-14 by lin-4 is also required for the timing of axon extension of the HSNs (29). In lin-4 loss of function animals, the HSNs do not extend their axons prior to the larval-adult transition, and adult axons have defective morphology. Therefore, lin-4 is required to temporally regulate extension of axons in two neuronal paradigms in C. elegans (29).

Later in neuronal development, miRNAs also control synaptogenesis and remodeling in C. elegans. For example, lin-4 targets lin-14 to remodel motor neuron synapses during the first larval stage L1, when motor neurons eliminate their synapses with ventral muscles and instead form connections with dorsal muscles. The timing of this change is regulated by the heterochronic genes lin-4–lin-14 (28) and mir-84–hbl-1 (45). mir-1 also plays important roles in synaptic function, by targeting the transcription factor MEF-2 to control neurotransmitter release at the neuromuscular junction (NMJ) (46). mir-1 also targets synaptic proteins neurexin (47), which in humans are two synaptic proteins that have been linked with defects in synaptic function associated with autism spectrum disorders (ASDs) (48). Elucidating these mechanisms in C. elegans may reveal conserved pathways and mechanisms underlying neurodevelopmental disorders.

Interestingly, miRNAs have also been implicated in the developmental decline of regenerative ability of the nervous system. The miRNA let-7 is only expressed very weakly when initial axonogenesis occurs, and onset of expression with age contributes to a reduced capacity for regeneration of the AVM neurons (30). This mechanism involves a developmentally regulated loop including the TRIM protein LIN-41, among other factors. The exciting discovery that suppression of let-7 could restore regenerative capacity (30) could be conserved in vertebrates, and indeed it has since been shown that suppression of Let-7 in primary cultured rat peripheral neurons increases their regenerative response (49).

Finally, the miRNA mir-71 was found to regulate the physiology of C. elegans non-cell autonomously from the nervous system (50). The authors showed that mir-71 expression in the AB lineage (nearly all neurons) is necessary and sufficient for lifespan extension of animals lacking a germline (50). The authors showed that neuronal mir-71 regulates the localization and activity of the FOXO transcription factor DAF-16 in the intestine which acts downstream of insulin-like signaling to regulate metabolism and stress responses (50–52). Such non-cell autonomous regulation of intestinal DAF-16 by mir-71 is via an, as yet, unidentified neuronal factor.

**Drosophila melanogaster**

MicroRNAs in the fruit fly, *Drosophila melanogaster*, have been extensively investigated in various developmental processes [reviewed in Ref. (53)]. miRNAs are critical for all aspects of neuronal development, from regulating neural stem cells to regulating the events that occur at the NMJ. For example, control of neuronal progenitor proliferation is fine-tuned by the highly conserved miRNA, mir-124, which has been shown in various organisms to regulate neuronal stem cells (54–57). In *Drosophila*, mir-124 targets anachronism (ana), an inhibitor of neuroblast proliferation. The absence of mir-124 results in decreased proliferative activity, which is coupled with an increase in ana expression (58). In addition to this, mir-124 is required for optimal regulation of dendrite growth and targets components of the retrograde BMP signaling pathway to regulate synaptic release at the NMJ (59).

Additional miRNAs that contribute to neuronal proliferation are the fly homologs of mammalian miR-92 and miR-200 – mir-92a/b and mir-8, respectively (60, 61). mir-92a is located in the intron and mir-92b in the 3’UTR of a putative DNA-binding protein, jigr1, and they suppress this host gene to regulate neural stem cell development to prevent premature differentiation (61). Additionally, mir-8 has been implicated in regulating neuronal proliferation but is expressed in a glial cell population ensheathing the optic lobe neuroepithelium (60). In the latter of these roles, mir-8 is required for the temporal and spatial control of EGFR pathway ligand, Spitz, which controls accurate neuroepithelial proliferation and neuroblast formation (60).

*Drosophila* has been used as a model to identify miRNAs that temporally and spatially control neuronal differentiation and specification. An example of this is with olfactory neuronal morphogenesis, which is associated with accurate miRNA function. Loss of core components of the miRNA biogenesis machinery, including Pasha or Dicer, results in abnormal olfactory neuron morphogenesis (62). The basis for these defects was defined by studies of the mushroom body neurons (MB), which mediate olfactory responses and comprise of four invariant subtypes of neurons in various insects (63–66). The generation of MB neurons requires tight post-transcriptional regulation of the BTB-zinc finger chronologically inappropriate morphogenesis (chinmo) in postmitotic neurons (66). This is achieved by a group of miRNAs that are cotranscribed from a single locus and comprise of mir-125, mir-100, and the highly conserved let-7, collectively referred to as the let-7-Complex (let-7-C) (67). The initial discovery of let-7 in C. elegans identified a heterochronic role for let-7, whereas in *Drosophila* let-7 expression is not enriched in early development, but upregulation of let-7-C is associated with a downregulation of chinmo (68). This suggests that the mechanism identified in C. elegans where let-7 regulates developmental timing functions in a different context in Drosophila to regulate the formation of MB neurons.

The involvement of miRNAs in dendrite growth is unclear, although a small number of studies in *Drosophila* have identified two miRNAs required for dendrite growth of sensory neurons. First, the miRNA, bantam, has been implicated in dendrite scaling by suppressing Akt kinase activity in nearby neurons and by regulating epithelial endoreplication (69, 70). Secondly, miR-9a (mammalian miR-9 homolog) acts from epithelial cells to fine-tune
dendrite growth. This is achieved by regulating the activity of a cadherin-domain containing putative G-protein-coupled receptor, Fmi, which functions to suppress dendrite growth (71). miR-9a also acts with miR-7 to control the development of sensory organs. In Drosophila, sensory organs develop from single organ precursor cells (OPCs), which are generated from clusters of cells expressing proneural genes. This process is temporally controlled by Notch signaling and two transcription factors that regulate proneural gene expression. These consist of Senseless, a positive regulator of OPCs, which is targeted by miR-9a, and a negative regulator, Enhancer of Split, which is targeted by miR-7 (72–74). Furthermore, miR-9a is also influenced by the RBA-binding protein, TDP-43, mutants of which display increased OPC cells coupled with decreased miR-9a expression (75). Although the exact requirement of TDP-43 in this process is unclear, genetic interaction assays suggest that SOP specification requires TDP-43 for accurate neuronal differentiation by influencing miR-9a activity.

MicroRNAs have also been shown to regulate the NMJ in Drosophila embryos and larvae. miR-8 and miR-289 are required to suppress activity-dependent synaptic growth by targeting genes involved in axon development and growth. miR-8 downregulates wingless, a presynaptic regulatory protein required for activity-dependent axon terminal growth at the NMJ (76). In this context, miR-8 regulates the timing of synaptic expansion to correlate with the growth of target muscles. Furthermore, miR-8 regulates the embryonic expression of two synaptic immunoglobulin superfamily cell adhesion molecules (IgCAMs), Fasciclin III (FasIII), and Neuroglan (Nrg) (77). Taken together, these studies lay a foundation for further study into the role(s) of miR-8 in presynaptic events, as well as the timing of synaptic assembly with neuron–muscle association.

Finally, miR-8 has also been shown to regulate apoptosis in the CNS of Drosophila (78). miR-8 regulates the expression of the transcriptional corepressor Atrophin to a particular threshold level. Loss of miR-8 results in increased atrophin levels and apoptosis; however, reduction of atrophin expression below the threshold set by miR-8 causes extra tissue being generated (78). Precise tuning of atrophin levels is, therefore, required to prevent neurodegeneration in the CNS of Drosophila.

miRNA REGULATION OF THE NERVOUS SYSTEM IN VERTEBRATES

Xenopus laevis

Neuronal development and function have been extensively studied in the Xenopus laevis tadpole [reviewed in Ref. (79)]. However, in contrast to other model organisms, the influence of miRNAs during neurodevelopmental processes has received less attention. Nevertheless, certain conserved miRNAs investigated in Xenopus offer new insights to their function. For example, similar to other model organisms, miR-124 regulates early neurogenesis. However, in Xenopus, miR-124 is expressed from the beginning of eye development where it plays an important role in regulating retinal neurogenesis in the optic vesicle and forebrain (57, 80, 81). In addition to this, miR-129, miR-155, miR-214, and miR-222 contribute to developmental timing of retinal progenitor cells by regulating the activity of the transcription factors, xotx2 and xvisx1, which are both required for promoting the late-stage progenitor cells to differentiate into bipolar neurons (82).

The highly conserved miR-9 is also required for neurogenesis along the anterior–posterior axis by targeting the transcription factor, hairy1, although its function varies from the hindbrain to the forebrain. In the forebrain, regulation of hairy1 by miR-9 influences proliferation of neural progenitor cells through Fgf8 signaling, but via Wnt signaling in the hindbrain (83). This suggests positional specificity regarding miR-9 function. Defects associated with the nonsense-mediated mRNA decay (NMD) pathway result in neurological disorders in humans (84, 85). Interestingly, in Xenopus, miR-128 has been shown to repress NMD by targeting the RNA helicase, UPF1, and the exon-junction cofactor, MLN51 (86). This process allows upregulation of specific mRNAs required for differentiating neuronal cells, which are normally targeted by NMD. Moreover, this mechanism is highly conserved in mammals and represents a dual mRNA regulatory network to maintain neuron development and function (86).

Danio rerio

Danio rerio (zebrafish) is a valuable model system that has been used to uncover neurodevelopmental functions for miRNAs, with the advantage that zygotic loss of miRNAs can be examined in the absence of maternal compensation mechanisms, since the zygote develops outside of the mother (87). Embryos carrying dicer mutations display severe developmental defects, including delayed embryogenesis, perturbed neurulation, and formation of brain ventricles, as well as ill-defined anatomical boundaries, such as the midbrain–hindbrain boundary (MHB) (87). However, interestingly the same study also showed that despite the gross morphological defects, gene expression, and neuron specification were maintained within patterned regions, such as the forebrain and hindbrain rhombomeres. Later stage neuronal differentiation, such as axon extension, was also severely affected by loss of dicer (87).

In zebrafish, miRNAs are expressed in neural cells throughout the different stages of development in addition to mature neurons. They can have ubiquitous or cell-specific expression patterns. Many conserved miRNAs are expressed at the same developmental timepoints as other vertebrates, for example, miR-9 and let-7 are expressed in both proliferating and differentiating cells (88). As in C. elegans, lin-28 and its downstream heterochronous genes let-7 and lin-4/miR-125b are expressed during development to coordinate cell proliferation (89).

In zebrafish hindbrain development, miR-107 stabilizes dicer levels, which maintains a specific level of miR-9 biogenesis to regulate optimal proliferation of neural progenitors (90). miR-9 inhibits proliferation at the MHB and hindbrain ventricular zone through targeting of proproliferation genes her5, her6/Hes1, and zic5 and then later also influences neuronal maturation by regulating elav3/HuC (91–93). Additionally, miR-9 overexpression causes a strong reduction in the MHB and cerebellum, as well as blurred somatic boundaries and altered cell fates, through downregulation of fgfr1 in the Fgf signaling pathway (91).

More recently, zebrafish hindbrain development has been used as a model system to uncover precise mechanisms of the miRNA-mediated mRNA decay pathway (94). In this study, a genetic screen
implicated cnot8, which was known to have deadenylase activity in polyA tail removal in mRNA turnover. Furthermore, the role of cnot8 in the Fgf signaling pathway is responsible for hindbrain dopaminergic neuron differentiation, and application of a drug inhibiting Fgf signaling partially restored the mutant phenotype (94). Zebrafish studies also elucidated the mechanisms through which morphine influences dopaminergic neuron differentiation, since maternal influence can be removed from developmental events. Morphine downregulates miR-133, which increases pitx3 expression thereby promoting dopaminergic neuron maturation (95). This study revealed important information relating to neural networks involved in drug addiction.

In the later stages of zebrafish neurodevelopment, miR-430 controls trigeminal sensory neuron migration. These sensory neurons arise from the neural crest and placodes, and their migratory journey of up to 120 μm requires the chemokine SDF1a and its receptor Cxcr4b. The border of SDF1a expression shifts continually to make a tightly regulated chemotactic path for neurons to migrate, and miR-430 is important for clearing SDF1a from the pathway that neurons have passed through (96).

Finally, in zebrafish embryos, miRNAs are also involved in dendritic spine formation and synaptogenesis. For example, knockdown of miR-153 caused a sevenfold increase in spontaneous body movement, and the synaptic protein SNAP-25, which is involved in vesicular exocytosis, was found to be the target (97).

**Mus musculus**

The earliest mammalian studies following the discovery of miRNAs in *C. elegans* quickly demonstrated the crucial nature of the class of non-protein-coding RNAs in mammals through the generation of mice carrying deletions for miRNA-processing pathway components including Dicer, Dgcr8, and Argonaute. Argonaute proteins are essential components of the RISC complex, facilitating translational inhibition or target mRNA cleavage, and Argonaute 2 mutants show an early and severe neurodevelopmental phenotype, with neural tube closure failing to occur (98). Deletion of another component of the miRNA-processing pathway, Dgcr8, causes microcephaly in mutant mice but is much less severe than loss of Dicer mutants (99). Under investigation is the potential importance of Dgcr8 in Di George syndrome, a multifaceted disorder where 30 genes including Dgcr8 are deleted, and has been associated with schizophrenia (100).

Loss of Dicer causes lethality in lower eukaryotes, such as *C. elegans* and *Drosophila* (101, 102). However, the neurodevelopmental consequence of Dicer loss in mice has been investigated in greater depth using conditional deletion mutants (103, 104). This led to further confirmation of both the spatial and temporal importance of miRNA-mediated pathways in multiple phases of mammalian CNS development. One study used conditional Dicer deletions generated with Emx-Cre (excitatory cerebral cortex neurons) and Nestin-Cre (all CNS neurons) mouse lines at different stages of embryonic cerebral cortex development showed that miRNAs are important for three major phases of cerebral cortex development: neuronal progenitor proliferation, neuronal migration, and differentiation (105).

Conditional mutants have been further used to confirm the importance of miRNAs in the progression of cerebral cortex development, with Cre-recombinase-driven deletion of Dicer under the control of Foxg1, Emx1, Nes, Nex, and CamkII promoters (106). These models have shown that in the early stages of cerebral cortex development, conditional deletion leads to a loss of neurons, either due to loss of the neural progenitor pool or increased apoptosis. Deletion of Dicer leads to neuron migration defects and impaired cellular differentiation, as well as cell fate changes and cortical lamination defects (106).

Conditional deletion of Dicer from the embryonic day 8 (E8) telencephalon causes a loss of radial glial progenitor markers, including nestin, Sox9, and ErbB2, which then results in an increase in basal progenitors and postmitotic neurons (107). Interestingly, increased apoptosis was also observed, and this was correlated with reduced expression of miR-9 and miR-124 (107), two miRNA families that have been widely implicated in brain development. Dicer conditionally deleted using the Nex-Cre promoter (targeting neurons of pyramidal fate) resulted in significantly smaller mouse brains, due to increased packing density of neurons, as well as abnormal neuron differentiation, but no defect in neuron production or cortical lamination (107). Conditional deletion of Dicer by an a-CaMKII-Cre in the embryonic forebrain resulted in microcephaly due to increased apoptosis, rather than neuronal migration defects, since lamination appeared unaffected. Reduced dendritic branching and dendritic spine development was also observed (103), along with ataxia and reduced life span. Interestingly, Dicer deletion in neural crest cells (by Wnt1-Cre) does not affect migration and early differentiation, but miRNA pathways appear to be required for the survival of peripheral nervous system (PNS) neurons, because in Dicer deletion mutants PNS neurons are lost after completion of migration and differentiation due to apoptosis (108, 109). Thus, while Dicer mutants all point toward dysregulated nervous system development, individual studies have reported slightly different mechanisms. Whether this is due to the timing of deletion, the promoter driving Cre-recombinase or which miRNAs are being lost due to blocking the processing pathway is not clear.

Dicer deletions have been useful in determining the overall impact of loss of miRNAs on gene regulation, but more information has been gained from studying the roles of specific individual miRNAs. The let-7 family, which was one of the first described in *C. elegans*, was subsequently also shown in the mouse to play a significant role in maintaining the balance in neuronal progenitor proliferation and neurogenesis, since TRIM32 (110) and SOX-2 (111) influence let-7 levels to maintain cells in a proliferative state. let-7b in turn regulates neural stem cell proliferation by targeting the stem cell regulators TLX and cyclinD1 (112).

The let-7 family is one of the most highly conserved and abundantly expressed miRNA families in the vertebrate brain and is also involved in balancing neural progenitor proliferation and controlling progenitor state (93). miR-9 regulates early progenitor proliferation in the mammalian brain through the transcription factors Hes1 (113), Foxg1, Elav2, Pax-6, as well as Gsh2 (114). Confirming its proliferative role, loss of miR-9 suppresses neural stem cell proliferation, through stathmin (115).

The miR-124 family is also conserved from *C. elegans* to humans. It is expressed by differentiating neurons in the subventricular zone of the developing mouse cortex, where it controls...
apical/basal progenitor progression (56). It is also important for continual production of neurons in the subventricular zone of the adult mouse brain (116). In neuronal differentiation, miR-124 is involved in a mechanism with the transcriptional repressor REST, whereby REST represses miR-124a and expression of neuronal genes in non-neuronal cells and neural progenitors. But at later stages, REST ceases to repress miR-124a, allowing non-neuronal transcript degradation and thus promoting neuronal differentiation (117). REST also has miR-124-binding sites in its 3′UTR, suggesting a complex regulatory loop exists (118). Another transcriptional repressor involved in the REST complex, MeCP2, also has predicted miR-124-binding sites in its 3′UTR (118), and MeCP2 mutation or copy number variant has been implicated in multiple neurodevelopmental disorders including X-linked intellectual disability and autism (119, 120).

Therefore, the above-mentioned in vivo studies suggest that miR-9 and miR-124 are major players in the regulation of cerebral cortex development, but in vitro studies have shown that miR-9 and miR-124 can drive the neurogenic program. When Yoo and colleagues (121) added miR-9 and miR-124 precursors to cultured neonatal foreskin fibroblasts, they were able to directly convert them to neurons expressing the mature marker MAP2, albeit at a conversion rate of <5%. Neuron conversion was improved remarkably by the addition of NEUROD2, ASCL1, and MYT1L, a combination that has previously been shown to produce neurons (122). Importantly, after 4 weeks in culture, the neurons expressed synaptic markers and displayed electrophysiological properties consistent with neurons (121). This exciting study suggests that miRNAs are not only involved in controlling gene expression in neurodevelopment, but could be considered among the master regulators of neurogenesis in mammalian cells.

Many other miRNAs have been implicated in the earliest stages of mammalian brain development and regulate important pathways in development and disease. For example, miR-130b has been shown to regulate Fmr1 expression, which is lost in the disorder fragile X syndrome. Fmr1 loss causes increased progenitor proliferation and altered neuronal fate specification (123). miR-135a2 regulates Wnt signaling in midbrain dopaminergic neuron proliferation in a regulatory circuit with Lmx1b (124), and in the mouse cortex, miR-134 promotes neural progenitor cell proliferation and counteracts apoptosis and differentiation (125). miR-34, another conserved miRNA, appears to be linked with neuron proliferation, because overexpression of mir-34 in human stem cells suppressed the expression of 136 neuronal progenitor genes that possess putative miR-34 target sites. Gene ontology showed that these genes are overwhelmingly involved in cell motility and energy production (126).

In addition to being crucial for neuronal progenitor proliferation, miR-9 and miR-124 are emerging as key regulators of neuron migration. miR-9, along with miR-132, represses Foxp2 to regulate radial migration in the developing mouse cortex. Ectopic expression of Foxp2 in the developing cortex was counteracted by increased endogenous expression of miR-9 and miR-132 (127). In a different mechanism, miR-124 and miR-22 regulate cell shape changes in migrating cortical neurons by controlling expression of doublecortin, a microtubule-associated cytoskeleton protein involved in cell shape remodeling through multipolar and bipolar phases in migrating neurons, through CoREST/REST (128). Recently, the miR-379-410 cluster was shown to regulate N-cadherin expression, a crucial factor in maintaining tissue structure in the developing cortex. Overexpression of these miRNAs in radial glial cells decreased N-cadherin expression, causing increased stem cell differentiation and migration (129). miR-128 in the brain regulates Phf6, which is a mutated gene in the disorder Borjeson–Forssman–Lehmann syndrome, and ectopic expression of miR-228 in the developing brain leads to neuron migration defects, neurite outgrowth, and electrophysiological changes (130).

Specific miRNAs exhibit increased expression in the latter stages of nervous system development in the mouse and are implicated in final differentiation, neurite extension, and synapse formation. These miRNAs include miR-134 (125, 131) and miR-132 (132–134). Also, miR-124 regulates RhoG, which is a major player in the control of axon and dendrite outgrowth and complexity, in mouse hippocampal neurons (135).

Very recently, expression of a miR-137 gain-of-function construct, first in cell lines, and then in the mouse hippocampal dentate gyrus, was shown to downregulate three well-known presynaptic proteins: complexin-1 (Cplx1), N-ethylmaleimide-sensitive fusion protein (Nsf), and synaptotagmin-1 (Syt1) (136). In vivo, this was accompanied by fewer synaptic vesicles and impaired hippocampal LTP, and impaired hippocampal dependent learning in behavioral testing. Excitingly, some of these defects were rescued by codelivery of a miR-137 sponge construct, which sequestered endogenous miR-137. These findings and rescue in the mouse brain are particularly promising, given the vast data implicating miR-137 SNPs in schizophrenia (136).

**Homo sapiens**

An estimated 70% of all miRNAs are expressed in the human nervous system [reviewed in Ref. (137)], although only a small number of miRNAs appear to be regulated during neuronal differentiation (138). Moreover, temporal and spatial distribution of miRNAs in human donor brains, as well as target genes associated with neurodevelopmental diseases have been identified (139). Despite this, ethical complications prevent in-depth mechanistic studies from occurring in humans. Therefore, expression of these conserved miRNAs, coupled with mechanistic studies from model organisms or cell lines, has allowed for neuronal miRNAs to be understood in greater detail [for reviews, see Ref. (140, 141)]. The majority of investigations in humans use cell lines or use screening-based approaches for miRNAs that are associated with populations of particular neurodevelopmental disease states. Cell line-based approaches, despite their in vitro limitations have characterized several miRNAs required for neuronal proliferation and differentiation. For example, overexpression of the highly conserved miR-9 promotes proliferation of neural progenitor cells in human embryonic stem cells (115). miR-9 along with miR-124 and miR-125b has also been associated with inducing human pluripotent stem cells to differentiate into neurons (142). Although described previously in *Drosophila* and in the mouse, miR-9 is a good example of an evolutionary conserved miRNA that contributes to various aspects of neuronal development.

Aberrant expression of miRNAs has been associated with different neurodevelopmental disorders, such as schizophrenia,
autism, Down syndrome, fragile X syndrome, and Rett syndrome [reviewed in Ref. (143–145)]. Determining if the aberrant expression of all of these associated miRNAs is simply the consequence of abnormal neuronal development or the cause of the disorder itself is challenging. Nonetheless, several studies have identified numerous aberrantly expressed miRNAs associated with bipolar and schizophrenic patients (146, 147). For example, a contributing factor in schizophrenia is decreased function of the N-methyl-D-aspartate (NMDA) receptor and delivery of NMDA receptor antagonists phenocopy the conditions associated with schizophrenia (148–150). Interestingly, miR-132 is downregulated in schizophrenic patients and has also been shown to contribute to the depolarization of the NMDA receptor (151, 152), suggesting that miR-132 may be a candidate for potential therapeutics. Defects associated not only with the expression of key miRNAs, but also at a genetic level have been implicated in schizophrenia. A genome-wide study of over 40,000 schizophrenic patients identified a SNP within the putative coding region of miR-137 resulting in decreased efficiency of miR-137 function (153). This is further supported by additional studies that have shown variation of miR-137 affects brain activation and function (154–156). Unlike examples of differentially expressed miRNAs, miR-137 suggests a direct genetic-miRNA association with schizophrenia.

Neurological diseases that give rise to ASDs, such as fragile X syndrome and Rett syndrome, display elevated and depleted miRNA expression [for detailed review, see Ref. (157)]. In addition to this, mutations associated with the miRNA machinery are thought to contribute to the progression of ASDs. For example, mutations in the RNA-binding protein, fragile X mental retardation 1 protein (FMR1), are associated with fragile X syndrome patients (158). In Drosophila, FMR1 functions as a RISC cofactor to maintain miRNA function (159). Another example is associated with Rett syndrome, which is caused by mutations associated with the methyl-CpG-binding protein 2 (MECP2) (160), which prevent nuclear miRNA processing by regulating the Drosha complex (161). Despite the importance of these proteins in human disease states, most mechanistic insights regarding FMR1 and MECP2 have come from investigations employing model organisms. Furthermore, these FMR1 and MECP2 studies show how mutations in key proteins associated with miRNA targeting or processing contribute to neurological diseases that give rise to autism.

Screening-based approaches to understand ASD-associated miRNAs involve postmortem analysis of autistic patients, as well as assessing circulating miRNAs in serum and plasma. This screening approach not only has led to a greater understanding of miRNAs associated with ASDs but also has potential to use selected miRNAs as non-invasive biomarkers for ASDs. Postmortem analysis of the cerebellar cortex identified 28 dysregulated miRNAs from 13 autism patients (162). This included miRNAs that are predicted to target the synaptic scaffolding protein, SHANK3, and the presynaptic cell adhesion protein, NRXN1, which are both associated with ASDs (163, 164). More recently, two studies carried out in live patients have identified 13 miRNAs in 55 children, as well as five miRNAs in 15 patients in China that are differentially expressed in serum and plasma in children with ASDs (165, 166). These investigations have also revealed the enrichment of predicted target genes of these differentially expressed miRNAs in various neurological pathways, suggesting a potential use for diagnosis and future therapeutic approaches.

**CONCLUSION**

Since the initial discovery of miRNAs being involved in developmental timing of larval development in C. elegans (6, 8, 9), small non-coding RNAs have been implicated in a multitude of biological processes. lin-4 and let-7 were identified in unbiased forward genetic screens, as was isy-6, the first miRNA shown to be involved in the nervous system (38). These genetic approaches enabled the identification of miRNAs with very specific roles during development. As such, these particular functions may have been overlooked when using reverse genetic techniques. However, waiting for such chance discoveries takes much time; therefore, other approaches are required to systematically drive miRNA discoveries forward. With the advent of temporal expression pattern analysis, sophisticated RNA sequencing and proteomic approaches, and miRNA prediction algorithms, the ability to move from a mutation in a specific miRNA to phenotype is a possibility using model organisms. Therefore, the meticulous scrutiny of miRNA expression patterns and screening for anatomical, functional, and behavioral phenotypes has proved fertile ground in the identification of roles for miRNAs in the brain.

In humans, the expression of a number of miRNAs has been correlated with neurodevelopmental disorders (143–145); however, the molecular mechanisms through which they act are not understood. Using model organisms as a discovery tool enables researchers to study the function of these miRNAs in depth. Due to the high degree of conservation between model organisms and humans, the elucidation of molecular mechanisms that control neuronal development using these models will help identify novel therapeutic approaches in the future.

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