

Review

Fine-tuning the brain: microRNAs

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ABSTRACT

The brain is of bewildering complexity and numerous genes and signaling molecules have been described that affect the architecture and functioning of specific neuronal circuits. Recent evidence from genome analysis revealed the existence of a large group of novel RNA molecules with unexpected properties. One such group is called microRNAs, which are small 21–23 nucleotides RNA molecules that are transcribed by the genome. However, they are not translated into proteins but rather control translation of coding mRNA. Particularly in the brain, numerous different microRNAs are expressed in a cell type specific fashion both during development and in adulthood. Aberrant microRNA expression has been implicated in several human diseases including CNS diseases. The aim of this review is to emphasize their role in the development of the brain and their function. In addition, we highlight recent findings on the evolution of mammalian microRNAs and their effect on steroid signaling in the brain.

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1. Introduction

A milestone in biological research was the completion of the DNA structure of the human genome in 2001 [40]. Rather surprising and for numerous researchers somewhat disappointing was the discovery that the human genome encodes 'only' 20,000–25,000 genes. It was estimated that only 1.5% of the genome consists of protein-encoding sequences and another 5% or so covered regulatory sequences and is transcribed into well-known RNA species like ribosomal RNAs and transfer RNAs. The vast majority, over 90%, was believed not to be transcribed and was collectively referred to as 'junk DNA'; a kind of immense playground for evolutionary processes. Eight years later this view on the human genome has dramatically changed. Novel technologies, like deep sequencing [65] and the use of DNA tiling arrays [10] revealed a highly dynamic transcription of the mammalian genome and it is now believed that over 80% of the DNA is transcribed into RNA. Numerous different RNA species have been described ranging from antisense transcription of over 50% of all coding genes [25] to complete novel families of regulatory RNA species like the PiWi RNA family [23]. In terms of biological function, most of these novel identified RNA species are largely unknown. The vast majority are non-coding; i.e. are not translated into a protein, suggesting that they execute their biological action via Watson & Crick base-pairing with specific target sequences, either with DNA or RNA.

An emerging group of non-coding RNAs are called microRNAs (miRNAs); small 21–23 nucleotide-long single-stranded RNA molecules. The first miRNA, *lin-4* was identified in *Caenorhabditis elegans* and reported in 1993 [44] to control developmental timing. However, 8 years elapsed before the first glimpse of the miRNA family became clear in mammalian species [39,42,43]. Based on deep sequencing techniques and on in silico predictions [5], the human genome is now believed to contain more than 1000 miRNA genes. Notably, the diversity of the miRNA repertoire increases with the organism's complexity: humans and other mammals have about four times more annotated miRNA genes compared to insects and nematodes, suggesting the role of miRNAs in the development of this complexity.

The biogenesis of microRNA is rather diverse. Approximately 50% of mammalian miRNAs are transcribed as a cluster as a pri-miRNA molecule that contains multiple hairpin-like structures. The pri-miRNA molecule is cleaved in the nucleus by the RNase Drosha, resulting in multiple pre-miRNA hairpin-like molecules that are actively exported to the cytoplasm by exportin. In the cytoplasm, the hairpin is further cleaved by a second RNase molecule called Dicer, resulting in an imperfect doublestranded RNA molecule of 22 bp. Finally, the doublestranded molecule is denatured and one strand, the mature miRNA, gets incorporated in a protein complex called RNA-Inhibiting Silencing Complex (RISC). The biogenesis of other miRNAs is different. Some of these are transcribed as part of protein-encoding pre-mRNAs, where they are typically located in intronic sequences and processed as part of the splicing machinery. Others are transcribed as independent transcription units. Transcription of most miRNAs is mediated by

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polymerase II, obviously including the intron-derived miRNAs. Some miRNAs, however, have been reported to be transcribed by polymerase III. For further information on miRNA biogenesis we refer to excellent reviews of Kim et al. [34,32].

RISC-miRNA complexes act as post-transcriptional gene regulators by binding to specific domains in the 3'-untranslated regions (UTRs) of target mRNAs, thereby inhibiting their translation. For binding to their mRNA target, perfect base pairing of at least seven nucleotides, called the seed region, at the miRNA 5'-end is crucial, while the remaining portion of the 3'-miRNA seems less important (for reviews see [3,4]). In humans, approximately 30% of all mRNAs, in particular transcription factors, are regulated by miRNAs [28]. Precisely how bound RISC-miRNA complexes inhibit translation is unknown at present but it has been suggested that proteins of the RISC complex interact with proteins belonging to the translation initiation machinery [36].

Numerous miRNAs are expressed in a cell-specific manner [41] and miRNAs activity has been associated with a number of biological phenomena, particularly in dynamic processes like embryonic development [21,31] and stem cell proliferation [29,61,63,73] and consequently, aberrant miRNA expression has been associated with diseases like cancer [15]. Also in the brain, miRNAs have been associated with neuronal plasticity. For example, miR-134 was found to regulate BDNF-stimulated synaptic plasticity by local translational inhibition of the lim-domain containing protein kinase 1 (Limk1), a regulator of actin filaments in spines [59]. Interestingly, miR-134 is derived from miR-379–410, a large cluster of brain-specific miRs. Transcription of this cluster is regulated by neuronal activity via myocyte enhancing factor 2 (Mef2, [20], a negative regulator of synapse numbers in adult hippocampal neurons that controls structural plasticity in striatum after cocaine exposure [56]). Recently, several excellent reviews have been published on the present status of our knowledge on miRNAs and neuronal plasticity and neuronal diseases [9,37,75]. In this review, we will summarize our present knowledge on the role of miRNAs in neuronal development, their role in neuro-endocrine signaling and briefly review our understanding of miRNA evolution in the mammalian brain.

2. MicroRNA and neuronal development

Numerous miRNAs have been found in neuronal tissues with a greater diversity than identified in other tissues, which might reflect the complexity and many different cell types found in the brain [5,41,33,50,60]. A considerable number of these miRNAs appear to be enriched [2] or even unique for neuronal tissue. For example, miR-124 and miR-128 are unique for neurons while miR-23, miR-26 and miR-29 are specifically expressed in astrocytes [9,62].

Brain development has been associated with highly dynamic and temporally regulated waves of miRNA expression, with specific groups of miRNA being expressed only at specific time-points during embryonic development of the nervous system [62,19,54,71]. miRNA gene expression profiling studies of cell-lines and stem cells induced to differentiate into a neuronal phenotype are in line with these *in vivo* findings. For example, P19 cells and embryonic stem cells, being exposed to neuronal phenotype-inducing agents like retinoic acid, start to express specific miRNAs, like miR-124 and miR-128, suggesting that these miRNAs play pivotal roles in neuronal differentiation [60,62,38]. Such a miRNA role is further highlighted by the use of transgenic animals in which Dicer, the RNase that is crucial for proper miRNA biogenesis, is removed in specific brain cells. For example, Dicer removal in the mouse during corticogenesis leads to microcephaly and changes in dendrite morphology and ultimately death around three weeks

after birth [16]. Similarly, deleting Dicer in Purkinje cells will ultimately lead to progressive cerebellar degeneration and ataxia [58]. In a genetic animal model for schizophrenia, reduced levels of DGCR8, another protein involved in miRNA biogenesis, lead to reduced miRNA expression, behavioural deficits and neuronal failure [64]. Clearly, these combined miRNA expression studies during brain development, and loss of function studies for proper miRNA biogenesis in neuronal tissues indicate not only their vital role in healthy neuronal development and function but also suggest a miRNA role in the pathogenesis of CNS diseases.

Undoubtedly the best studied miRNA in neuronal development is miR-124. As mentioned above, miR-124 is expressed only in neurons and not in any other cell type [60,38]. Interestingly, when overexpressed in non-neuronal HeLa cells, the gene expression profile shifts from an immature cervix cell into a neuronal phenotype [49], suggesting that miR-124 downregulates mRNAs directing cells into a non-neuronal phenotype. In line with this notion are the findings that miR-124 overexpression in differentiating P19 cells promotes neurite outgrowth while inhibiting miR-124 has the opposite effect. Indeed, a number of direct miR-124 targets, with anti-neuronal activities, have been identified. Firstly, miR-124 downregulates REST, a transcriptional repressor of numerous neuronal genes including miR-124 itself [14] and the anti-neuronal small C-terminal domain phosphatase 1, which is a component of the REST complex [69]. Secondly, miR-124 binds to the 3'UTR of PTBP1 mRNA, a repressor of alternative pre-mRNA splicing in non-neuronal cells [53]. Thirdly, in differentiating neuronal stem cells in the subventricle zone, miR-124 targets Sox9, a factor that is necessary for glial cell generation and prevents neuron formation [12]. Thus, by downregulating Sox9, miR-124 directs differentiating stem cells into a neuronal phenotype and blocks glia formation. Finally, miR-124 targets directly glucocorticoid receptor (GR) mRNA thereby reducing cellular responsiveness towards glucocorticoids [70], adrenal hormones that inhibit proliferation and differentiation of neuronal progenitor cells [66,72]. Thus, it appears that miR-124 acts as a master-switch that turns down the activity of key genes maintaining cells in a non-neuronal state, thereby promoting the activation of differentiation program that leads to a neuronal phenotype (see Fig. 1).

3. MicroRNA and neuro-endocrine signaling

Since the discovery of the miRNA gene family and their action on cell proliferation, extensive research has been devoted to their

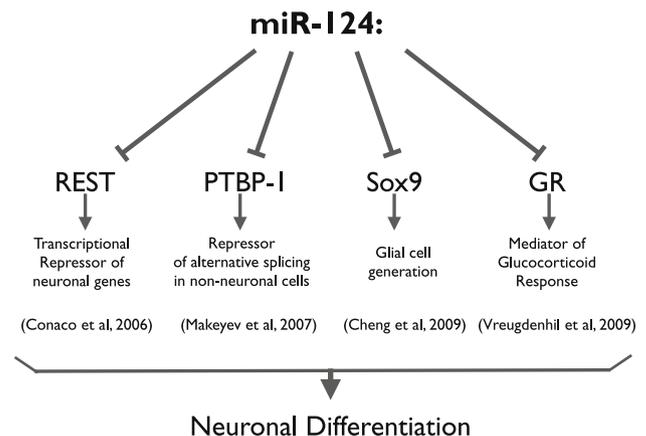


Fig. 1. miR-124 acts as a neuronal differentiation-inducing factor. MiR-124 has been shown to repress the activity of REST [14], BTBP [53] and Sox9 [12], all proteins known to antagonize the formation of neuronal cells during development. miR-124 also binds to the 3'UTR of GR mRNAs suggesting that reduced GR protein levels are necessary for proper neuronal differentiation. For further details: see main text.

role in cancer. In endocrine-related cancers, like breast cancer, several studies appeared showing aberrant miRNA expression that is related to estrogen [13,35,51,76,1] or glucocorticoid signaling [57]. In pituitary adenomas, miR-15 and miR-16, both of which are known to down-regulate the anti-apoptotic bcl-2 gene, were expressed at reduced levels, a finding that was correlated with greater tumor diameters [8]. Undoubtedly, given their pivotal role in cell proliferation, miRNAs in neuroendocrine tumors will prove to be a fruitful field for future biomedical research.

Recent evidence also indicates a prominent miRNA role in endocrine and neuro-endocrine signaling. In *C. elegans*, environmental-stimulated progression of development is regulated by the nuclear hormone receptor DAF-12, a homologue of mammalian vitamin D receptors that is activated by steroidal ligands. Recently, it became clear that ligand-activated DAF-12 directly activates transcription of let-7 miRNA family members that subsequently downregulate hbl-1, a gene product that is crucially involved in developmental progression. On the other hand, inactive DAF-12 represses let-7 miRNA family members, resulting in hbl-1 arrest of developmental progression. It therefore appears that these miRNAs are environmental-(in)activated molecular switches of developmental larval stages in *C. elegans* [7]. In *Drosophila*, the steroid hormone ecdysone regulates metamorphosis and development by activating a nuclear hormone receptor, the ecdysone receptor (EcR) and this process also involved EcR-induced downregulation of miR-14 expression, while miR-14 itself downregulates EcR expression [68]. A similar negative feedback loop between the orphan nuclear receptor TLX and miR-9 has recently been reported to be a key regulator in neuronal stem cell differentiation, in which miR-9 promotes neuronal differentiation by repressing TLX translation and TLX expression induces neuronal stem cell proliferation and represses miR-9 expression [77] (see Fig. 2).

miRNAs have also been implicated in the neuroendocrine control of diuresis. Mice exposed to 2% saline in their drinking water, a well-known model to study c-fos induced vasopressin expression in the hypothalamus, showed altered expression of a number of microRNAs in the paraventricular nucleus (PVN). Interestingly, one abundantly-expressed miRNA, miR-7b, was increased by saline

exposure in the hypothalamus and was found to target c-fos in vitro thereby reducing c-fos protein levels [45]. Thus, a model emerged from these studies in which increased osmolarity lead to a dual increase in c-fos and miR-7b expression; c-fos will activate vasopressin expression and miR-7b will subsequently reduce c-fos activity thereby functioning as a safety valve by preventing c-fos-mediated transcriptional overshoot.

A number of miRNAs have been identified that control the expression of hormone receptors thereby regulating cellular responses towards hormone exposure. For example, the 3'UTR of Estrogen receptor mRNA is targeted by miR-206 [1] and miR-221/222 [76]. Both these miRs are expressed in breast cancer cells and it has been suggested that upregulated miR-221/222 expression is responsible for the observed resistance towards anti-estrogen therapy [76]. In brain, the expression of these miRNAs does not seem to be enriched [41] and miR-206 has been reported to be muscle-specific [74]. However, besides breast cells, the miR-221/222 cluster is upregulated in glioblastoma cells [22,52], which might have implications for reported tamoxifen therapy of this type of cancer [55]. Recent evidence indicates that glucocorticoid signaling is also controlled by different and cell-specific miRNAs. One of these miRNAs, miR-18, has been suggested to bind to the GR 3'-UTR in the PVN thereby reducing GR protein levels [67]. GR mRNA-miR-18 interaction has been suggested to underlay increased susceptibility towards stress as miR-18 is expressed higher in the PVN of Fischer 344 rats, a strain that exhibits exaggerated acute-stress induced release of corticosterone [18,17], compared to Sprague–Dawley rats. However, miR-18 is broadly expressed in most adult mammalian tissues (see e.g. [41] and in vivo miR-18 levels are lower than what is needed to repress GR protein levels in vitro [70]). Therefore, the physiological significance of miR-18 in glucocorticoid signaling in the brain remains to be determined. Interestingly, miR-124a, a well-characterized brain-specific miRNA (see above), also reduces GR protein levels in vitro, and in vivo miR-124a levels are higher than what is needed for reducing GR protein in vitro, suggesting physiological relevance of miR-124a expression for glucocorticoid signaling. Particularly, in the hippocampus and in the first 2 weeks of the neonate life, miRNA-124a expression is highly dynamic [70]. This period is known as the stress hyporesponsive period (SHRP), during which neonates fail to generate an elevation in glucocorticoids upon exposure to a stress, and which coincides with a period of neuronal differentiation. As reviewed above, proper neuronal stem cell differentiation is regulated by miRNA-124a and thus it may be part of the mechanisms by which stressors are relatively ineffective during SHRP. Thus, it will be of interest to study a possible causal relationship between fluctuating GR and miRNA-124 expression.

Given the fact that many miRNA are expressed in a cell type-specific manner, in particular in the brain [41], and that miRNAs are believed to particularly target transcription factors, such as nuclear hormone receptors [28], it is likely that this novel family of non-coding RNA is extensively involved in the fine-tuning of the response by which cells, in particular neurons, react to hormone exposure. Given the many miRNAs and the young history of miRNA research it is also likely that the findings described above are just a small tip of the iceberg and that many exciting discoveries are in front of us.

4. Evolutionary dynamics and innovation in miRNA genes

The initial small RNA sequencing efforts in humans and mice revealed several hundred miRNA genes that were generally well conserved among vertebrates and were expressed at high levels. Further deep sequencing of small RNAs from human and chimpanzee brain identified a number of additional miRNA genes that were

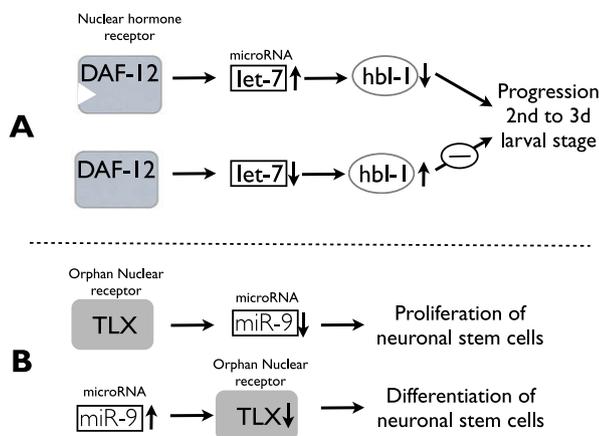


Fig. 2. Examples of miRNAs in (neuro)endocrine signaling. A: in *C. elegans*, favorable environmental factors result in the synthesis of steroidal compounds (black triangle) that activate the nuclear hormone receptor DAF12 thereby inducing the expression of miRNA let-7. Subsequently, let-7 binds to hbl1, a repressor of larval stage 3, resulting in the progression of the 2nd to 3rd larval stage. On the other hand, unfavorable conditions lead to DAX12 receptors that actively repress let-7 expression resulting in increased hbl1 protein levels and arrest at the 2nd larval stage [7]. B: in mammals, the orphan nuclear receptor TLX induces miR-9 expression leading to proliferation of neuronal stem cells. As a feedback mechanism, miR-9 represses TLX protein formation leading to differentiation of neuronal stem cells [77]. For further details: see main text.

expressed at lower levels and showed less conservation between species – many miRNA were conserved only between primates and some were even species-specific, suggesting the existence of recently evolved miRNA genes [6]. The fact that these non-conserved miRNAs are expressed at low levels supports the model of miRNA acquisition developed by Chen and Rajewsky. They proposed that newly emerging miRNA genes initially need to be expressed weakly and in specific tissues or developmental stages in order to minimize the negative impact these miRNAs might have on the organism's fitness, and to allow elimination of deleterious miRNAs binding sites by natural selection before the level of miRNA expression can be elevated [11]. One implication of this miRNA acquisition model is that many of the recently emerged and lowly expressed miRNAs genes most likely have no biological function yet, and some of them would disappear in the course of evolution, while only a small fraction would develop a function. Indeed, the analysis of miRNA divergence patterns revealed that miRNAs with low expression levels evolve rapidly and that almost 30% of annotated human miRNA genes show little evidence of selective pressure [47]. At the same time, low levels of expression and conservation do not necessarily indicate that such miRNAs do not have a function. Rather, the existence of taxon- and species-specific miRNAs opens up the intriguing possibility of miRNA's involvement in the development and modification of gene regulatory networks that may allow development of more complex systems [26], including differences in brain function between human and primates [6]. The challenge that lies ahead is the identification of such 'innovative' miRNA genes within the pool of mainly neutral miRNAs.

5. Future perspectives

Since the discovery of miRNAs in mammalian systems in 2001, over 4500 papers have appeared describing novel miRNAs, their biogenesis and their role in specific biological processes, their targets and their involvement in disease processes. Despite this volcanic eruption of miRNA research several important issues remain. Firstly, in silico experimentations suggest that a single miRNA is able to target the 3'UTR of a few hundred different mRNAs. For example, one of the best characterized miRNAs, the brain-specific miR-124a, is predicted to bind to almost 1300 conserved targets (targetscan 5.1, [24,46], while only a couple of these targets, such as REST [14], SOX9 [12] and the glucocorticoid receptor [70] have been tested experimentally. Clearly, novel technology, enabling the simultaneous detection of many miRNA targets, as described by Karginov et al. [30] or crosslinking and immunoprecipitation followed by high throughput sequencing (CLIP-seq; [27,48], are needed to address this issue. Secondly, in relation to the first point, present identification of miRNA targets depends on in vitro experimentation in which specific miRNAs with putative targets, cloned in reporter constructs, are co-expressed in cell-lines. In addition, identification of disease-related microRNAs is mainly based on large-scale gene expression studies and provides mainly an association of microRNAs with regulatory molecules and do not reveal any physiological relevance. Clearly, manipulation of endogenous microRNAs by in vivo loss and gain of function studies, for example as conducted by Cheng et al. [12], is required to address this issue. Also, how does the resulting stochastic ratio miRNA/target of in vitro experimentation relate to what is present in vivo? Thirdly, miRNA are believed to act as master switches, turning on or off molecular network programs that direct a cell to proliferate, to differentiate or to change its morphology. Questions therefore arise about the molecular and environmental factors that regulate miRNA expression. Using the different in vivo animal models including *C. elegans*, zebrafish and rodents, and the numerous available tools,

such as lentiviruses and anti-miRNAs to manipulate miRNA expression in vivo, these questions can now be addressed.

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