Mini-review

What are the roles of microRNAs at the mammalian synapse?

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The modification of neuronal connections in response to stimuli is believed to be the basis of long-term memory formation. It is currently accepted that local protein synthesis critically contributes to site-restricted modulation of individual synapses. Here, we summarize recent evidence implicating miRNAs in this process, leading to altered dendrite morphogenesis and synaptic plasticity. Second, we discuss findings in non-neuronal systems about how RNA-binding proteins can modulate miRNA–mRNA interactions, and how these mechanisms might apply to neurons. Finally, we review recent findings that P-bodies may be important sites for miRNA action at the synapse.

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The reaction of specific neural circuits to stimuli and their modifications by experience at the cellular and molecular level during memory formation is the underpinning of current research in molecular neuroscience. There are two distinct forms of memory depending on the duration: short-term memory (STM) and long-term memory (LTM) [12]. Stable modifications in neuronal connections and in the strength of synaptic activity are believed to underlie LTM. Long-term potentiation (LTP) and long-term depression (LTD) serve as molecular models to study memory, and are a measure of increased or decreased synaptic strength, respectively. In analogy to memory, LTP has two temporal phases, an early phase and a late phase [12]. Early phase LTP is manifested by quick post-synaptic alterations in the geometry of the postsynaptic density and in the strength of synaptic activity and are a measure of increased or decreased synaptic strength, respectively. In analogy to memory, LTP has two temporal phases, an early phase and a late phase [12]. Early phase LTP is manifested by quick post-translational modifications of pre-existing proteins at the synapse, whereas the late phase requires new gene expression and protein synthesis. In addition to global protein synthesis in the cell body, new proteins are also produced in dendrites [39]. This observation suggests a very attractive model where site-restricted modulation of the protein composition at a single synapse in response to various repetitive stimulations would lead to synaptic plasticity [39,40]. Obviously, a prerequisite for local translation is the presence of miRNA [10] and translation machinery in the vicinity of synapses [39]. Several hundred transcripts have been identified in synaptoneurosomes or isolated dendrites ([35] and references therein), however only a few have been reliably shown to localize to dendrites by in situ hybridization (ISH) in cultured neurons or in brain slices. Dendritically localized miRNAs are found in ribonucleoprotein particles (RNP) or neuronal RNA granules that are transported to distal dendrites by molecular motors, presumably in a translationally silent state [6,12]. Thus, RNA-binding proteins (RBPs) might have dual roles, one as localization determinants and another as translational regulators. A fascinating and new addition to the mechanisms of translational regulation at the synapse are small non-coding RNAs, in particular microRNAs (miRNAs). In this review, we summarize recent data that demonstrate the importance of miRNAs in regulating protein synthesis in the vicinity of the synapses that undergo plastic changes during formation of memory. We also discuss possible mechanisms for this miRNA regulation and the putative sites of action in dendrites.

miRNAs are small non-coding RNA effectors of post-transcriptional regulation that are 19–22nt in length. They are transcribed in the nucleus as long primary transcripts, which are further processed in the nucleus and the cytoplasm to yield mature miRNA duplexes [16]. One strand of the duplex is then loaded into the RNA-induced silencing complex (RISC), of which the Argonaute (Ago) proteins are a major component [34]. In mammals, the vast majority of miRNAs are partially complementary to target mRNAs, in some cases only in the 6–8nt long 5′-seed region of the miRNA, and repress their targets by either blocking translation or causing their destabilization. To date, there are only a few examples of miRNA-mediated cleavage of target mRNA through perfect base pairing, as is more commonly seen in plants, in mammals [3]. Since the requirement for target complementarity is only partial, this means that one miRNA can potentially have hundreds of targets [3]. The discovery of miRNAs provoked a frenzied attempt to inventory the expression pattern of miRNAs in different organisms and organ systems. The brain expresses a disproportionately large number of tissue-specific or -enriched miRNAs, suggesting important roles in brain-specific functions [24,26]. Currently, the miRNA database,
miR-base, reports 706 miRNAs in humans, 547 in mouse and 286 in rat (http://microrna.sanger.ac.uk/sequences), with new miRNAs constantly being identified. Recent deep sequencing of miRNAs cloned from different mouse brain regions found a total of 329 miRNAs expressed [26], therefore, more than half of all presently known mouse miRNAs are expressed in the brain. Many miRNAs are temporally regulated, with the expression of several peaking in the adult animal, suggesting roles for those miRNAs in the mature brain [24]. In addition, the presence of a large number of primate-specific miRNAs expressed in the brain has raised the interesting possibility that miRNAs have played a role in the evolution of brain function [5].

One way to analyze brain-expressed miRNAs has been to determine their subcellular localization in mature neurons. The Kosik laboratory used laser capture techniques to isolate the somatic and dendritic compartments of mature cultured hippocampal neurons, followed by multiplex real-time PCR to identify both the mRNA and miRNA components of these compartments. Using stringent criteria, only 4 miRNAs (miR-292-2p, miR-26a, miR-26b, miR-25) were significantly enriched in dendrites when comparing the ratio of abundance to the somatic compartment [25]. The dendritic localization of miR-26a was additionally confirmed by ISH and was reported to repress translation of the dendritically localized mRNA, MAP2. In an alternative approach, Lugli and colleagues [29] biochemically purified synaptoneurosomes and used a microarray to identify the miRNAs present (Table 1). The novelty here came from the identification of not only mature miRNAs, but also the pre-miRNAs. Coupling this with the presence of Dicer and Ago2 in this biochemical fraction [29] and references therein), raises the intriguing possibility that mature miRNAs may be processed from their precursor in very specific subcellular domains, such as individual synapses.

Some of the best indications in vivo for the role of miRNAs in differentiated neurons, however, have come from region-specific knockouts of Dicer, an essential component for miRNA biogenesis. Loss of Dicer in the forebrain results in ataxia, microcephaly and decreased lifespan. In Purkinje cells, inactivation of Dicer leads to cerebellar degeneration and development of ataxia ([12] and references therein). In addition, mice heterozygous for Dgcr8, another component required for miRNA biogenesis, exhibit reduced dendritic complexity, altered dendritic spine morphology and cognitive deficits [38]. These phenotypes all point to an essential role for the miRNA pathway in mammalian neuronal survival and function. In Drosophila, pasha (Dgcr8 homolog) and Dicer-1 mutants were identified in a forward genetic screen for genes involved in the targeting of projection neuron dendrites in the antennal lobe to higher brain centers, indicating a role for this pathway in the formation of correct neuronal connections [4]. It remains to be seen whether this role is conserved in mammals, but it seems plausible given the severity of the knockout phenotypes in mammals. In addition to the roles characterized in vivo, miRNA mis-regulation has also been linked to a number of psychiatric disorders and neurodegenerative diseases, including Tourette's syndrome, Rett's syndrome, Parkinson's disease and Alzheimer's disease [8].

The current challenge is to elucidate the biological functions of individual miRNAs in neurons. That miRNAs are essential for neuron differentiation and development is well established [24], however, their activity in mature neurons and, in particular, at the synapse is still not well characterized. Recently, several important studies have pioneered functional analysis of individual miRNAs in mature post-mitotic neurons, uncovering roles in dendrite morphogenesis, synaptogenesis [17,43] and synaptic plasticity [36,37].

During development, the specific pattern of arborization of dendrites determines the connectivity of a neuron. This process is influenced by activity and is critical to neuronal function [45]. It is in part regulated by activation of gene expression through a number of transcription factors. Among their myriad of target genes, activity-induced transcription factors also up-regulate a number of miRNAs [17,43].

Expression of miR-132 is induced by the transcription factor cAMP response element-binding protein (CREB) in response to neuronal stimulation in developing neurons and consequently effects a change in neuronal morphology [43]. Induction of miR-132 is dependent on NMDA receptor activity and leads to increased levels of the mature miRNA, both in the soma and dendrites. The consequent down-regulation of the miR-132 target p250GAP, a GTPase activating protein, leads to enhanced neurite outgrowth [43]. In a similar fashion, miRNAs from the miR379-410 cluster are up-regulated by the activity-dependent transcription factor myocyte enhancing factor 2 (MEF2) in response to neuronal stimulation, some of which then effect dendritogenesis [17]. In particular, miR-134, in addition to its role in regulating spine morphology in mature neurons (discussed below), regulates dendritic elaboration through down-regulation of the translational repressor Pumilio2 in response to neuronal activity [17]. This demonstrates an interesting interplay between translation control factors, miRNAs and transcription factors. Additionally, both studies provide a link between the transcriptional programs initiated in response to activity, and the post-transcriptional events controlling translation of pre-existing miRNAs that are affected by miRNAs and translational regulators.

Behavioral responses, including learning and LTM formation, require regulated synthesis of new proteins at synapses in Drosophila as in mammals [1,40]. Recent identification of miRNAs and mRNAs in distal dendrites near synapses lend weight to tempting theories pertaining to a local miRNA-mediated repression of targets involved in synaptic plasticity. Indeed, in a groundbreaking study, Ashraf et al. reported that synaptic protein synthesis occurs in Drosophila antennal lobes at the sites of the formation of stable memories in response to conditioned training of odor stimuli paired

<table>
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<th>Dendritic miRNA</th>
<th>Method</th>
<th>Target in neurons</th>
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<tr>
<td>miR-134</td>
<td>ISH</td>
<td>LIMK1</td>
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<td>miR-26a</td>
<td>ISH</td>
<td>MAP2</td>
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<td>miR-292-2p, miR-26b, miR-25</td>
<td>Laser capture and multiplex RT-PCR</td>
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<td>let7, miR-128</td>
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<td>miR-132</td>
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<td>miR-200c, miR-339, miR-342, miR-466, miR-425, miR-182, miR-350, miR-183, miR-351, miR-297</td>
<td>Microarray (synaptoneurosomes)</td>
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<td>miR-138</td>
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<td>miR-218, miR-9</td>
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<td>miR-29a, 2miR-7, miR-137, miR-335, miR-376b, miR-98</td>
<td>Microarray (synaptoneurosomes)</td>
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<td>miR-370, miR-9, miR-124</td>
<td>ISH</td>
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a Top 10 enriched miRNAs in synaptoneurosomes only.
b In situ hybridization (ISH) performed on cultured neurons except where indicated, on brain sections.
with electric shock [1]. To visualize synaptic protein synthesis, a fluorescent reporter containing the CaMKII 3′-UTR was expressed in transgenic lines. Both olfactory training and acetylcholine receptor stimulation of explanted brains produced increased dendritic transport and localization of this reporter together with CaMKII expression [Fig. 1]. In another recent report, the fragile X mental retardation protein (dFMRP) was shown to be involved in LTM formation via the activation of the mTOR (mammalian target of rapamycin) signaling pathway (Fig. 1).

The second miRNA, miR-138, was identified as a brain-specific, activity-regulated miRNA that is enriched in the synaptoneurosom fraction and localizes to distal dendrites in hippocampal neurons [37]. Alteration of miR-138 levels in hippocampal neurons showed similar spine phenotypes as observed for miR-134. Bioinformatic analysis suggested several candidate mRNAs from which, only one, Acyl protein thioesterase 1 (APT1), was validated in this study as a miR-138 target in neurons. APT1 is an enzyme that catalyzes the removal of palmitate modifications, and therefore its levels can affect membrane anchoring of many post-synaptic proteins. The authors indirectly demonstrate that repression of APT1 mRNA translation via miR-138 might prevent palmitoylation of the APT1 substrate, G protein α 13 (G_13). Membrane-bound G_13 acts as a RhoA activator, which in turn negatively regulates dendritic spine growth (Fig. 1) [37] and references therein. It will be interesting to see whether the action of miR-138 or some of the other dendritically localized miRNAs are also controlled by synaptic activity, and what further functions will be found for such miRNAs.

While miRNAs are now widely accepted as regulators of gene expression at the post-transcriptional level, their mode of action is still controversial [16,27]. Current models have been built on conflicting observations that miRNAs may act either at the step of translation initiation or elongation. Furthermore, there exist compelling data showing that miRNAs can induce target mRNA degradation [16,27]. The gaps in the experimental data leading to discrepancies in proposed concepts have been recently discussed [16].

A completely unexplored territory in the miRNA field has emerged only recently with the findings that RBPs might be important for miRNA functioning [7,22,42]. These proteins were not identified as core RISC/miRNA components, but their association appears to be critical for modulating the RISC/miRNA activities. It was observed in mammalian cell lines that miRNA-associated targets may shuttle between polyribosomes and certain “storage granules”, like processing bodies (PBs) or stress granules (SGs), depending on cellular conditions and the cell type [27]. Hence, a miRNA target might undergo a cycle of (reversible) repression and activation, suggesting the need for a regulatory mechanism.
This is particularly relevant in neurons, given the observations of activity-dependent de-repression of targets discussed previously.

The first example of a player that reverses miRNA repression came from work by the Filipowicz group [7]. A member of the embryonic lethal abnormal vision (ELAV) protein family, ELAV1 (also known as HuR), is required to release cationic amino acid transporter 1 (CAT1) mRNA from miR-122 repression in response to amino acid starvation in hepatocarcinoma cells. Under favorable conditions, CAT1 mRNA localizes to PBs in a miR-122-dependent manner. Upon stress, however, CAT1 mRNA is released from PBs into the cytoplasm to become translated in a process that requires ELAV1 binding to the AU-rich region in the 3′-UTR. Their great achievement is that they succeeded in visualizing an endogenous mRNA in PBs. Currently, we lack such “model” mRNAs to study their association with PBs in primary cells, probably due to a high mRNA turnover in PBs. To overcome this problem, many studies have been using overexpression of reporters containing miRNA-targeted 3′-UTRs.

The second RBP found to counteract mRNA-mediated silencing, is Dead end 1 (Dnd1) [22]. Dnd1 was initially described as a protein required for germ cell viability, since its mutation in mice causes germ cell loss accompanied by testicular germ cell tumors [22] and references therein. In a reporter-based screen, Dnd1 was found to elevate the expression of two tumor suppressor genes (LAT52 and p27) bearing functional miRNA-binding sites for miR-372 and miR-221, respectively [22]. In contrast to somatic cells in zebrafish, where miRNAs reduce nanos and trd mRNA levels, Dnd1 is able to protect these mRNAs from degradation in germ cells. The effect of Dnd1 is dependent on binding to a U-rich region close to the miRNA-binding sites where it may sterically block their access.

The latest and unexpected addition to the modes of miRNA regulation is provided by a recent report demonstrating the stimulatory effects of a miRNA on protein translation [42]. The basis for this interesting discovery was the initial observation that both Ago2 and FXR1 binding to AU-rich elements (AREs) in tumor necrosis factor α (TNFα) mRNA are required to increase its translation upon cell cycle arrest [42] and references therein. The TNFα 3′-UTR contains three tandem AUUUA motifs, two of which are flanked by sequences complementary to the seeds of miR-369-3. The authors showed that miR-369-3 represses TNFα translation in proliferating cells, whereas it enhances translation in arrested cells. The FXR protein thereby acts as the key molecular switch from miRNA-mediated repression to activation by binding to the ARE of TNFα mRNA only upon cell cycle arrest. This same mechanism was shown in the same paper to also apply for two other miRNAs. Thus, the cycling of activation-repression rounds could be a more general mechanism for miRNA function, which is under the control of RBPs.

All data concerning the mechanisms of miRNA-mediated regulation have so far come mostly from systems other than neuronal cells. Although the reversal of miRNA repression of localized mRNAs at stimulated synapses (CaMKII and LimK), has been documented [1,36, the exact molecular machinery behind this process is not understood. In light of the latest findings where RBPs regulate miRNA functions, it is very likely that such regulatory events occur at mammalian synapses as well. The first candidates are the ELAV proteins (HuB, HuC, and HuD), some of whose expression is restricted to neurons [32]. ELAV/HuD protein was previously implicated in memory formation as its levels undergo a sustained up-regulation in the hippocampus of trained mice in spatial discrimination and learning tasks [32]. Consistent with this, ELAV/HuD is recruited to dendritic spines upon glutamate receptor activation in cultured hippocampal neurons and associates with neuronal mRNAs encoding proteins implicated in synaptic plasticity [41]. Dnd1 protein is highly expressed only in the developing brain, and mice with genetically disrupted Dnd1 are viable, with no obvious neurological deficiencies [22] and references therein. Therefore, Dnd1 may not be the major determinant in miRNA-regulated cognitive functions, but it may still be involved in some miRNA-regulated processes during neuronal development. And lastly, FXR1 is a known interacting partner of FMRP, whose participation in miRNA functioning is still an unresolved issue [20].

A common trait of miRNA-dependent regulation is the involvement of ARE or U-rich sequences in miRNA-targeted transcripts. ARE-containing mRNAs (TNFα) are specifically transferred from polyosomes to PBs upon translation inhibition by the ARE-binding proteins of the tristetraproline family [18]. Conversely, ARE-binding RBPs relieve miRNA repression to re-enter translation (see above). Thus, the ARE-binding proteins, which have long been thought to determine mRNA stability or degradation, might execute this function at least in part by regulating the mRNA subcellular localization possibly in concert with bound miRNAs [21].

It is now widely accepted that cytoplasmic mRNAs can be stored and silenced in RNA granules, via translation repressor proteins and miRNAs [31]. However, where and how miRNAs operate on their target mRNAs is still an open question. The first hints came from studies on miRNAs and RISC in non-neuronal cells showing that Ago proteins localize to distinct cytoplasmic foci together with markers for PBs [14,16]. PBs were originally identified as cellular sites of mRNA decay that harbor exonucleolytic (Xrn1) and decapping enzymes (Dcp1, Dcp2), decapping activators (RNA helicase Rck/p54), factors of non-sense-mediated decay (Upf1, Upf2, Upf3) and other auxiliary proteins (GW182/TNRC8A, CCRA4-NOT deadenylase) [14,31]. Genetic studies also revealed a much closer relationship between miRNAs and mRNA degradation and repression in PBs than expected, as mutations in the Drosophila orthologues gawky (GW182), Me31B (Rck/p54), dDcp1 and dDcp2 (Dcp1 and 2), and the ccrl/twin-not) complex (CCRA4-NOT, disrupt miRNA-mediated translational repression and miRNA-mediated mRNA degradation in flies [14]. In mammalian cells, the GW182 protein family consists of three proteins, TNRC8A (GW182), TNRC6B and TNRC6G, all of which localize to P-bodies and are all required for miRNA function [15,19,28,30]. TNRC6A also directly interacts with Ago2 [15]. Furthermore, the localization of miRNAs and their targets in PBs [27] is another strong indication that PBs are potential sites of miRNA activity.

In the Drosophila nervous system, a range of established PB components (dDcp1, Upf1, Me31B/Rck) and Ago2 were observed not only in the cell body of cultured Drosophila sensory neurons, but also in dendrites [2]. Surprisingly, PBs and Ago2 extensively colocalized with components of distinct RNA granules, namely dFMRP and Staufen. In contrast to flies, however, immunostaining and dual color videomicroscopy of rat hippocampal neurons showed no significant overlap of Dcp1 and Rck with markers for transport RNPs (Stau1, Stau2, and Barentsz), suggesting a different interaction between transport RNPs and PBs in rodent neurons [46].

In another report, Cougot et al. demonstrated that dendritic PB-like structures labeled by GW182 contain Ago2 and endogeneous miRNAs (miR-128, let7), as well as a let7-target reporter in either hippocampal or hypothalamic neurons [13]. In addition, the authors detected a high degree of co-localization between GW182 and their targets in PBs [27] is another strong indication that PBs are potential sites of miRNA activity.

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different fractions would be required. Nonetheless, dendritic PB stability does seem to differ from that of HeLa PBs [14,31]. Photobleaching experiments showed that Dcp1 marked granules have a low rate of recovery, which contrasts with the rapid exchange detected previously in HeLa cells. In neurons, the efficiency of Ago2 recovery is higher than for Dcp1, although it is still below HeLa cell values (e.g., difference of ~20% for dendrites). Remarkably, the Dcp1 exchange rate in neurons is greatly improved upon stimulation with NMDA [27]. In addition, BDNF treatment of neurons induced the translocation of PBs into dendrites and the release of most of the Ago2 from the RNPs [13]. These results show that synaptic activation can change the dynamics and composition of PBs and suggests that stimulation of neuronal activity may contribute to localized derepression of translation.

In another study, Zeitelhofer and colleagues observed that both PB and Ago2 particles disassemble following neuronal stimulation with NMDA, glutamate or BDNF, supporting a model where synaptic PB and Ago2 particles disassemble following neuronal stimulation derepression of translation.

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