Identification of Novel microRNA Regulatory Proteins in Neurons Using RNAi-Based Screening

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Introduction

The proper functioning of the brain relies on the precise connection of billions of neurons. During the processes of learning and memory formation, specific synapses have to be modulated while neighboring contacts need to stay unchanged. This synaptic plasticity requires *de novo* protein synthesis in the participating synaptic compartments (Sutton and Schuman, 2006; Bramham and Wells, 2007). The local translation of preexisting mRNAs in dendrites is one way to ensure tight spatiotemporal regulation of protein expression in a highly synapse-specific manner.

During the past few years, a number of studies have assigned an important role for the regulation of local mRNA translation in neurons to microRNAs (miRNAs), a class of small noncoding RNAs (Bicker and Schratt, 2008). miRNAs exert a repressive effect on gene expression by binding to partially complementary sequences within the 3′ untranslated region (3′UTR) of target mRNAs. This repression leads to an inhibition of productive translation from the respective transcripts (Bartel, 2004). Owing to their mode of action, miRNAs represent an excellent way to regulate gene expression posttranscriptionally in a tight spatial and temporal manner. Consequently, they are involved in a great variety of cellular processes, including differentiation, metabolism, and cell death. In the brain, miRNAs play a crucial role at different stages of neuronal development and maturation (Fiore et al., 2008).

In order to achieve repression of translation, miRNAs recruit a multiprotein complex to the target mRNA: the so-called miRNA-associated RNA-induced-silencing complex (miRISC). Previous studies identified several important regulators of miRNA function, among which are either core components of the miRISC (e.g., Ago, GW182, Rck/p54, MOV10) (Chu and Rana, 2006; Chendrimada et al., 2007; Peters and Meister, 2007; Banerjee et al., 2009) or other regulatory proteins not directly associated with RISC (e.g., Dnd1, HuR) (Bhattacharyya et al., 2006; Kedde et al., 2007). Strikingly, all studies that screened systematically for miRNA regulators were performed in nonneuronal cells, leaving open the possibility that critical neuron-specific regulators of miRISC function remain to be identified. This possibility is particularly intriguing because such regulators could couple synaptic stimulation to miRNA-dependent control of local translation.

In this chapter, we describe a large-scale screening approach for testing the involvement of neuronal RNA-binding proteins (RBPs) in miRISC function. We present evidence that neuronal miRISC function relies not only on some of the RBPs previously identified in nonneuronal systems, but also on additional proteins that could allow miRNA function to adapt to the special needs of this highly specialized cell type to adapt.

Setting Up a Screening Approach for Regulators of miRNA Function in Neurons

In order to identify miRNA regulators in neurons, we set up a large-scale RNA interference (RNAi) screening experiment. We postulated that the knockdown of important effector proteins in neurons should relieve miRNA-mediated translational inhibition. We used the brain-specific miR-134 as a paradigm, since we had recently demonstrated an important role for this miRNA in the regulation of local mRNA translation in neurons. In mature hippocampal neurons, miR-134 localizes to dendrites, where it inhibits the translation of the Lim-domain-containing kinase 1 (LimK1) mRNA, thereby acting as a negative regulator of dendritic spine size (Schratt et al., 2006). The very same miRNA was later shown to regulate dendritic outgrowth by fine-tuning protein levels of the translational repressor Pumilio2 (Pum2) (Fiore et al., 2009).

For the RNAi screening experiment, we decided to analyze the function of miR-134 using a Luciferase reporter assay. We selected a reporter gene containing an miR 134 target 3′UTR downstream of the Luciferase coding sequence. The reporter-transcript we used harbors the 3′UTR of a newly identified miR-134 target gene that, in comparison with the other miR-134 targets (Limk1 and Pum2), is more strongly repressed by miR-134, making this a suitable readout for a large-scale RNAi screen.

The miRISC is formed by a few core components, such as Argonaute proteins and RCK/p54, and a number of associated factors, e.g., the fragile X mental retardation protein (FMRP) and the Vasa intronic gene (VIG) protein (Caudy et al., 2002), all of which are RBPs. In addition, RBPs like Dnd1 and HuR have been shown to interact with the 3′UTR of the target mRNAs, thereby modulating miRNA activity in an indirect way (Bhattacharyya et al., 2006; Kedde et al., 2007).

RBPs appear to be the key players of the miRNA effector machinery. Therefore, to study regulators of miRNA function in neurons, we decided to focus on this class of proteins. Using small interfering RNA (siRNA) technology, we planned to knock

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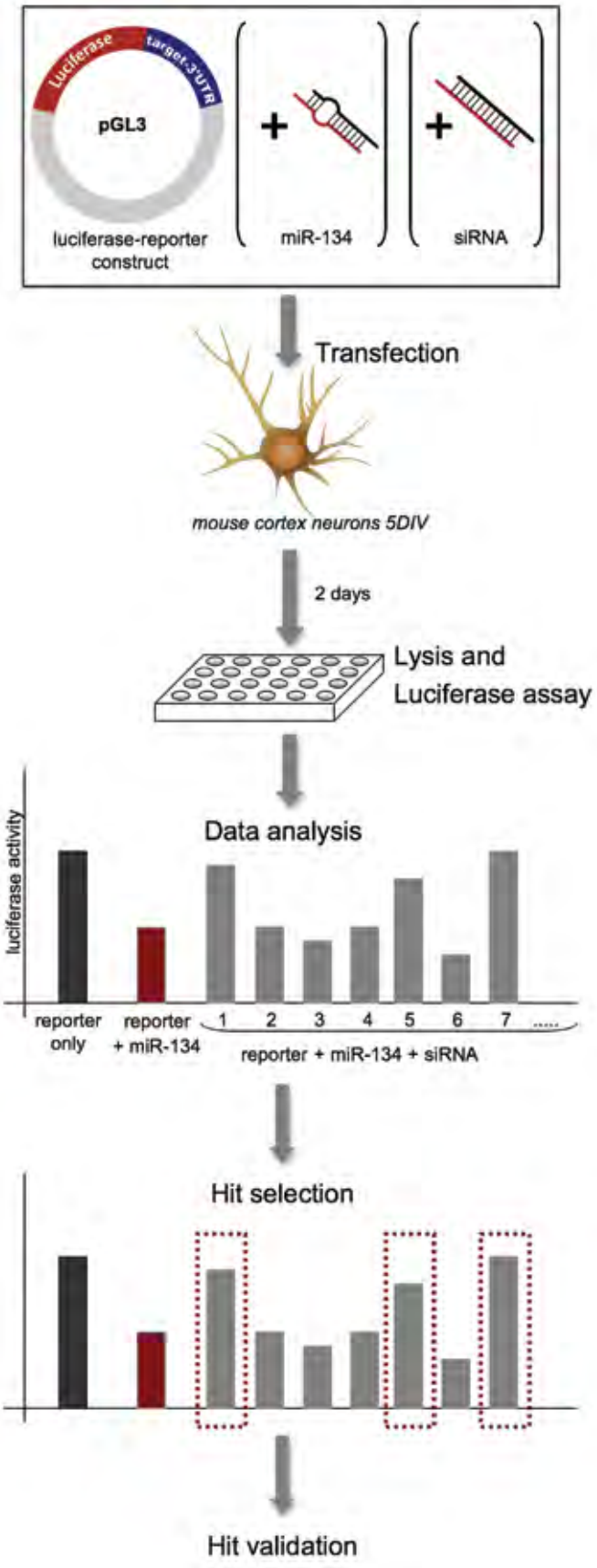

Figure 1. Flowchart of the RNAi screening experiment to identify regulators of miR-134 function.

down each neuronal RBP individually and assess its importance for miRNA-mediated translational repression in neurons using the described Luciferase reporter assay. Based on a previous *in situ* hybridization study (McKee et al., 2005), we listed all RBPs that had been shown to be expressed in postnatal mouse forebrain (~300) and ordered a custom siRNA library with three individual siRNAs for each candidate gene (Ambion, Austin, TX).

In initial experiments, the functionality of our approach was proven by knockdown of the RISC protein GW182 in neuronal cultures, which efficiently interfered with miRNA-134–mediated repression of the Luciferase reporter. For the actual screening experiments, 5 days *in vitro* (DIV) mouse primary cortical neurons were transfected with the miR-134–responsive Luciferase-reporter construct, together with the mature miR-134 duplex and an siRNA targeting one of the RBP genes. Luciferase assays were performed 2 d after transfection. Each condition was transfected in duplicates, and the entire screen was repeated twice.

Hit Validation

miR-134

For hit selection, we applied a threshold where at least two out of three different siRNAs for each targeted gene were able to relieve the miR-134–mediated repression by at least 50% in all three runs. Applying this threshold, the screen identified 10 RBPs that are required for miR-134–mediated repression of the target gene. Among the hits are known components of the miRNA pathway (GW182, RCK/p54) as well as RBPs that have not yet been implicated in miRNA function and therefore might be important specifically in the neuronal system.

To test the efficacy of the siRNAs, we cloned constructs for overexpression of a green fluorescent protein (GFP)–tagged version of the candidate RBPs. Those constructs have been cotransfected, together with the siRNAs, into

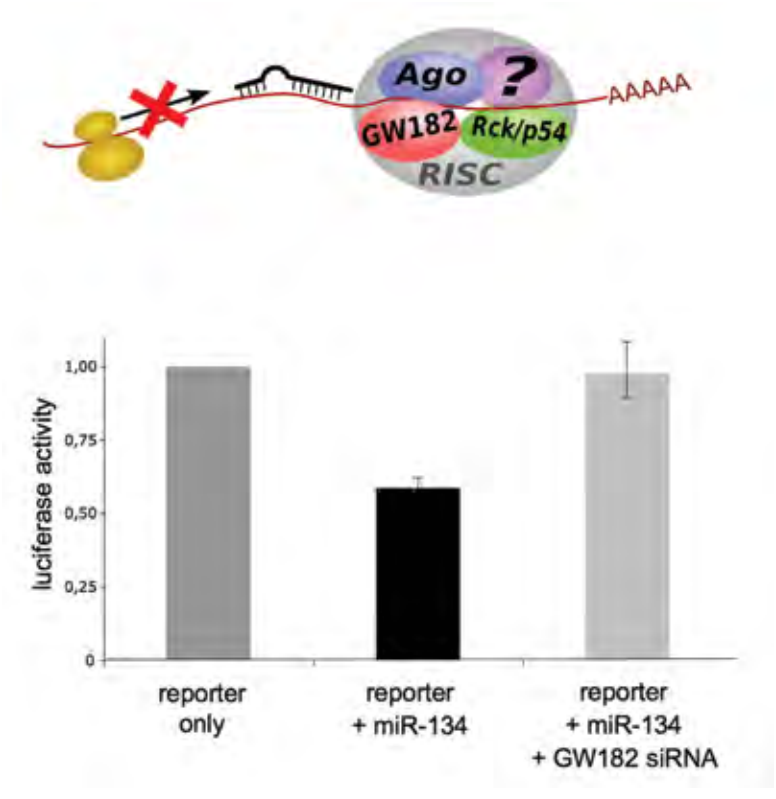

Figure 2. Knockdown of critical RISC proteins leads to the release of miR-134–dependent repression of reporter gene expression. Top: Schematic of the miRNA-associated RISC complex assembled on a target mRNA. RISC components that have been shown to be required for miRNA-mediated repression are indicated. Bottom: Results from a Luciferase reporter assay in cortical neurons using a GW182-siRNA in the context of miR-134 duplex RNA. Bars represent the mean of three independent experiments.

Hek293 cells, and their efficacy was assessed by immunoblotting using a GFP-specific antibody.

Next, we decided to validate the positive hits using several variations of the reporter assay. First, we verified whether the observed effects were indeed the result of impaired miR-134 function or whether the siRNAs affect 3′UTR–dependent translation in a more general fashion. To test this hypothesis, we repeated the Luciferase assay for the identified candidates using a Luciferase reporter construct with a mutated miR-134 binding site that does not show significant repression by miR-134. By doing so, we could discriminate between effects that result from a specific interaction with miR-134 and more general effects on mRNA translation that might arise by reason of either binding motifs for the candidate RBPs in the Luciferase reporter mRNA or direct contacts to the cap structure.

Our results indicated that knockdown of the candidate RBPs leads to an increased expression of the Luciferase reporter not only for the construct harboring the miR-134 binding site but also for the reporter-transcript with the mutated binding site. Therefore, the identified RBPs appeared not only to affect miRNA-134–mediated repression but to play a more general role in terms of translation regulation. This finding is not too surprising, however, because previous studies have shown that the miRISC components GW182, Ago2, and RCK/p54 behave in a similar fashion.

Next, we investigated whether the identified RBPs are general regulators of miRNA function in neurons, or whether some are specific for the studied miR-134–target interaction. To this end, we studied the effect of the candidate siRNAs in Luciferase reporter assays with different combinations of synaptic miRNAs and targets. We tested the two other validated miR-134 targets, LimK1 and Pum2, which allowed us to determine whether the RBP modulates miR-134 function in the context of a specific target gene (i.e., via direct interaction with the 3′UTR) or whether it is able to modulate miR-134 activity in general (i.e., by contributing to the miR-134–specific RISC). We found that the knockdown of our candidate RBPs interfered with miR-134–mediated translational repression independently of the target reporter construct. Thus, we concluded that the identified RBPs seem to play a more general role in miR-134–dependent translational control.

miR-138

Finally, we analyzed whether or not the RBPs also regulate the function of other dendritic miRNAs. The brain-enriched miR-138 is present at synaptic sites and functions, similar to miR-134, as a negative regulator of dendritic spine size. This effect is mediated, at least in part, by translational downregulation of its target, acyl-protein-thioesterase 1 (APT 1) (Siegel et al., 2009). In order to test whether our candidate RBPs are involved in the regulation of miR-138, we tested the respective siRNAs in the context of miR-138–mediated repression of APT 1. For all RBPs tested, the knockdown showed very similar effects

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on miR-138 and miR-134 function. Therefore, we concluded that the identified RBPs likely regulate the function of neuronal miRNAs in a more general way rather than in an miRNA-specific or target-specific manner. This finding, however, does not exclude the possibility that the function of the candidate RBPs may be modified in the context of a specific miRNA–target interaction.

We reasoned that RBPs that are able to regulate miRNA function at the level of the mature miRNA likely reside in the cytoplasm. To gain insight into the subcellular localization of our candidate RBPs, we transfected the described GFP-fusion proteins into hippocampal neurons and assessed the distribution of the GFP-signal using confocal microscopy. The results revealed that the majority of the candidate RBPs showed a pronounced or exclusive signal within the nucleus, matching their described biological function during mRNA splicing or nuclear export.

This observation raises the question: Why were these RBPs required for miRNA function in our RNAi screening setup? Because we transfected mature miRNA duplex, which bypasses processing, we can rule out the option that the nuclear localized RBPs affect miRNA function by simply blocking the first miRNA processing steps in the nucleus. Since we know that most of the candidate RBPs are involved in RNA export or RNA splicing, a possible scenario could be that these RBPs play a significant role in the proper transport or processing of mRNAs encoding important regulator proteins of miRNA function, e.g., Argonaute proteins or GW182. Alternatively, these RBPs might be present in the cytoplasm at very low levels, which we were unable to detect. Additional experiments beyond the scope of this study are needed to discriminate between these possibilities.

Experimental Outlook

Interestingly, a subset of our candidate RBPs was expressed throughout the whole neuron, including the most distal dendritic branches. These candidates are particularly promising because they might play a direct role in the regulation of miRNA function

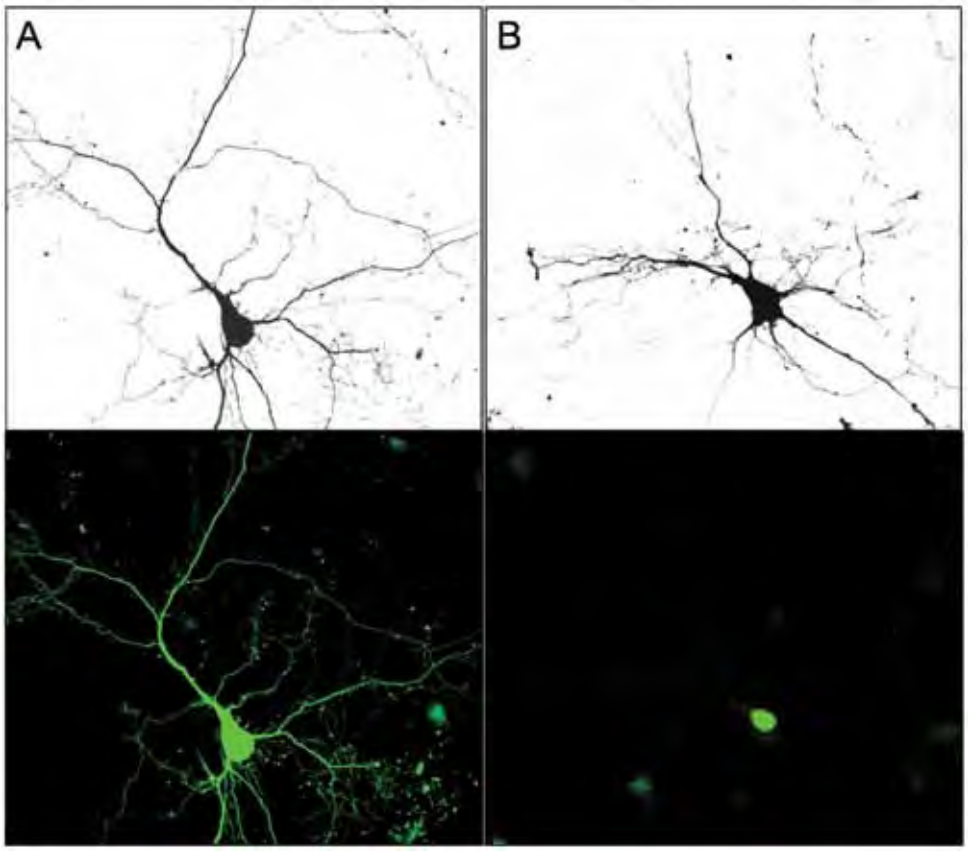

Figure 3. GFP-fusion proteins of two selected RBPs identified in the screen were expressed in hippocampal neurons. Whereas the RBP in panel ***A*** is expressed ubiquitously within the neuron, the RBP shown in ***B*** is restricted to the neuronal nucleus.

at the synapse. Therefore, we will focus on this set of candidates in future studies investigating the underlying mechanism and biological significance of neuronal function.

As a first step in our experimental efforts, we are interested in finding out whether the candidate RBPs directly associate with RISC. To this end, we will overexpress the RISC core protein Ago2 in Hek293 cells, together with the GFP-tagged RBPs, and perform co-immunoprecipitation experiments to reveal possible interactions. In addition, we will study a direct interaction of the RBPs with either the miRNA and/or the target transcript by performing pull-down experiments.

On a functional level, we will assess the importance of the candidate proteins for neuron maturation and dendritic spine plasticity. In the Luciferase experiments, knockdown of the candidate RBPs comparably impaired miR-134 and miR-138 function. Both miRNAs are known to negatively regulate the size of dendritic spines and, in the case of miR-134, promote dendritic outgrowth (Schratt et al., 2006; Fiore et al., 2009; Siegel et al., 2009). Since the selected candidates have been shown to localize to dendrites and synaptic sites, one can surmise that they regulate miR-134 and miR-138 function in the context of spine maturation and dendritic branching. Thus, we plan to knock down the RBPs in neurons under conditions of elevated miR-134/miR-138 activity to see whether we can counteract the neuronal phenotypes of miR-134/miR-138 overexpression.

Conclusions

We believe that it is feasible to perform a large-scale RNAi screening experiment in primary mouse neurons. We have already identified a small number of RBPs necessary for miRNA-mediated translational repression in neurons that so far had not been associated with miRNA function in other cell types. Thus, it is likely that neuron-specific miRNA regulators exist. They could prove to be critically involved in the modulation of miRNA-regulated local protein synthesis at synaptic sites and might present a new way to couple changes in neuronal activity to the regulation of protein levels at the synapse.

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