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Identification of Axon-Enriched MicroRNAs Localized to Growth Cones of Cortical Neurons

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Abstract

There is increasing evidence that localized mRNAs in axons and growth cones play an important role in axon extension and pathfinding via local translation. A few studies have revealed the presence of microRNAs (miRNAs) in axons, which may control local protein synthesis during axon development. However, so far, there has been no attempt to screen for axon-enriched miRNAs and to validate their possible localization to growth cones of developing axons from neurons of the central nervous system. In this study, the localization of miRNAs in axons and growth cones in cortical neurons was examined using a “neuron ball” culture method that is suitable to prepare axonal miRNAs with high yield and purity. Axonal miRNAs prepared from the neuron ball cultures of mouse cortical neurons were analyzed by quantitative real-time RT-PCR. Among 375 miRNAs that were analyzed, 105 miRNAs were detected in axons, and six miRNAs were significantly enriched in axonal fractions when compared with cell body fractions. Fluorescence *in situ* hybridization revealed that two axon-enriched miRNAs, miR-181a-1* and miR-532, localized as distinct granules in distal axons and growth cones. The association of these miRNAs with the RNA-induced silencing complex further supported their function to regulate mRNA levels or translation in the brain. These results suggest a mechanism to localize specific miRNAs to distal axons and growth cones, where they could be involved in local mRNA regulation. These findings provide new insight into the presence of axonal miRNAs and motivate further analysis of their function in local protein synthesis underlying axon guidance.

Keywords

microRNA; axon; growth cone

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Additional Supporting Information may be found in the online version of this article.

Introduction

Localization of mRNAs to subcellular compartments has been recognized as a fundamental mechanism to enable local protein synthesis and to regulate protein sorting with precise spatial and temporal control (Martin and Ephrussi, 2009). In neurons, the regulation of mRNA localization and local protein synthesis in response to neurotrophins, axon guidance factors, and neurotransmitters is necessary for axon guidance, regeneration, and synaptic plasticity (Steward and Schuman, 2001; Piper and Holt, 2004; Sutton and Schuman, 2006; Bramham and Wells, 2007; Lin and Holt, 2007). Localized mRNAs are regulated by mRNA-binding proteins, such as fragile X mental retardation protein, cytoplasmic polyadenylation element-binding protein, and zipcode binding protein 1, which traffic along axons and dendrites to localize their bound mRNAs into growth cones and synapses by binding cis-acting target sequences of mRNAs, often in the 3' untranslated regions (UTRs; Bassell and Kelic, 2004; Richter, 2007; Bassell and Warren, 2008). This specific localization system mediated by the trans-acting factors, mRNA binding proteins, plays a pivotal role in control of translation and metabolism of mRNAs needed for diverse neuronal functions.

Recent studies suggest that sequence-specific regulation of localized mRNAs is also controlled by non-coding RNAs that regulate mRNA translation and/or stability (Schratt et al., 2006; Muddashetty et al., 2011). MicroRNAs (miRNAs), 17- to 25-nucleotide (nt)-long small RNAs, act as additional trans-acting factors to regulate translation and metabolism of mRNAs (Eulalio et al., 2008; Fabian et al., 2010). Biogenesis of miRNAs is composed of two steps: processing of gene-transcribed pri-miRNAs into ~70-nt-long pre-miRNAs by the RNase III Drosha, located in the nucleus, followed by additional processing of pre-miRNAs to mature miRNAs by another RNase III, Dicer, in the cytoplasm (Bushati and Cohen, 2007; Ghildiyal and Zamore, 2009). Mature miRNAs bind 3' UTR target sequences in mRNAs to control translational repression and mRNA decay for regulation of protein expression (Eulalio et al., 2008; Fabian et al., 2010). Several recent studies suggest that in the nervous system, miRNAs play important roles in dendritic branching, spine morphology, and axonal pathfinding. Conditional, CNS-specific *Dicer* knockout mice show reduced dendritic branch elaboration, increased spine length, and axon tract abnormalities (Davis et al., 2008). Furthermore, Schratt et al. (2006) reported that a particular miRNA, miR-134, localizes to dendrites to regulate local synthesis of LIM kinase 1 (*Limk1*), which in turn controls dendritic spine development in hippocampal neurons. In the case of axons, the RNA-induced silencing complex (RISC), a critical component regulating mRNA metabolism and protein expression mediated by miRNAs, has been shown to localize to axons *in vitro* and *in vivo* (Hengst et al., 2006; Murashov et al., 2007). A recent study has identified a subset of miRNAs present in axons of sympathetic neurons (Natera-Naranjo et al., 2010); however, so far, no study has screened for miRNAs present in axons of developing cortical or hippocampal neurons. Of interest, profiling analysis has identified numerous mRNAs within axonal growth cones under developmental regulation (Zivraj et al., 2010). Recently, we showed that miR-134 and *Xlimk1* mRNA colocalized in axonal growth cones and that miR-134 is necessary for the regulation of protein synthesis-dependent growth cone attraction of *Xenopus* spinal neurons (Han et al., 2011). In another recent study, miR-9 and

miR-19a were reported to be present in axons and growth cones to regulate axon growth (Dajas-Bailador et al., 2012; Zhang et al., 2013). Taken together, these studies motivated the current work to identify axonal miRNAs localized to growth cones of cortical neurons, which may play important roles in regulation of axon guidance during brain development.

In this study, we identified miRNAs enriched in axons using a quantitative real-time RT-PCR (qRT-PCR)-based screen of axonal fractions obtained by cultured cortical neuron balls, a method that allows for efficient spatial separation of large amounts of purified axons. Fluorescence *in situ* hybridization (FISH) analysis in cultured cortical and hippocampal neurons validated the axonal localization of two of the miRNAs, miR-181a-1* and miR-532, that were enriched in the axonal preparations. These axonal miRNAs are localized clearly to axonal growth cones in contrast to only background levels of cell body-enriched miRNAs in growth cones. Moreover, their function as miRNAs *in vivo* was confirmed by coimmunoprecipitation with Ago2, a major RISC component. Our findings suggest that there is a specific mechanism for trafficking of miRNAs to growth cones that may regulate mRNA translation and metabolism necessary for axon extension and guidance.

Materials and Methods

Neuron Ball Culture and miRNA Analysis

Neuron ball cultures were prepared as described previously (Sasaki et al., 2010). Briefly, mouse cortices were dissected from E16 embryos. Hanging drops (neuron balls) containing 10,000 cells per drop were maintained for 3 days inside the top cover of 100-mm dishes that contained water on the bottom dish to maintain humidity. “Neuron balls” were then mechanically placed on poly-L-lysine (PLL)-coated dishes, containing Neurobasal media supplemented with GlutaMax and B27 (NB + GM + B27; all from Invitrogen), at 5-mm intervals. After 1 day, cytosine β -D-arabinofuranoside hydrochloride (AraC) was added to kill dividing cells to minimize the content of glial cells in the neuron ball culture. Neurites from neuron balls extended up to 1–2 mm over 5 days. The portion of the neuron balls containing cell bodies was collected by suction using sawed-off pipette tips (about 1 mm diameter), and the remaining axons were collected with cell scrapers. Total RNA was purified from the axonal and cell body fractions using the mirVana miRNA Isolation Kit (Ambion) with RNAqueous-Micro column (Ambion) and quantified using fluorometry with the RiboGreen reagent (Invitrogen). These RNA samples were analyzed by TaqMan miRNA Assay (Applied Biosystems), a qRT-PCR-based assay system using 384-well plates. At first, 5 ng of total RNA was reverse transcribed by 375 types of multiplex looped primers for miRNAs and then preamplified by Taq-Man PreAmp Kit for 14 cycles. For quality control to check for possible cross-contamination between the cell body and the axonal fractions, we used single-plex RT-PCR for snoRNA135, which is a type of small nucleolar RNA. We used only preamplified cDNA samples for which $Ct_{\text{axons}} - Ct_{\text{cell bodies}} [Ct(\text{Ax} - \text{CB})]$ for snoRNA135 was above 10 ($2^{10} = 1024$), which means that snoRNA135 in axons were over 1000 times less abundant than in cell bodies. This indicated that contamination of axon fractions from the cell body is less than 0.1%. Typically, we recovered about 20–30 ng of total RNA from the remaining distal axonal fractions obtained from 1200 neuron balls. The cell body fraction contains proximal axons; however, as 100–150 μg total RNA (over 5000

times more than the axonal fraction) from the cell body fraction was typically recovered, axonal RNA contamination in the cell body fraction can be considered as minor and thus negligible. The preamplified cDNA from three biological replicates was analyzed using the TaqMan Array Rodent MicroRNA Cards (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Ct values of all miRNAs were calculated from raw data using the 7900HT Fast System Detection Software version 2.3 (Applied Biosystems). The qRT-PCR for miRNAs detected 192 types of miRNAs in cell bodies, and 159 paired Ct values expressing both axons and cell bodies were obtained. As larger Ct values tended to have larger fluctuation, we set 33 as the cutoff value to obtain reliable results. We also omitted two paired Ct values due to higher standard errors ($SE > 2$). As a result, 105 paired Ct values of axons and cell bodies were processed using RealTime StatMiner (Intergenomics) for statistical analysis (Supporting Information Table S1).

Immunoprecipitation for qRT-PCR

Mouse cortices and hippocampi (postnatal day 21) were dissected and homogenized in immunoprecipitation buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, and 1% NP-40 supplemented with protease and RNase inhibitors] using plastic pestles and 25-g syringes. Protein lysate (1 mg) was incubated with 10 μ g mouse anti-AGO2 antibody (Abnova) for 2 h at 4°C. Protein G Sepharose (Roche Applied Science) was added for another 2 h, followed by several washes with immunoprecipitation buffer. miRNAs were extracted from beads using Trizol (Invitrogen). miRNAs were quantified as described previously (Muddashetty et al., 2007), with some modifications. A poly(A)-tail was added, and miRNAs were *in vitro* transcribed using the qScript™ MicroRNA cDNA Synthesis Kit from Quanta Biosciences. This cDNA was used as template for SYBR1 Green qRT-PCR (Roche Applied Sciences) using a miRNA-specific primer as described previously (Ro et al., 2006).

FISH Detection of miRNAs

Dissociated mouse cortical and hippocampal neurons from E16 embryos were cultured for 4 days in NB + GM + B27 and fixed with 4% paraformaldehyde in phosphate-buffered saline. After prehybridization, the fixed cells were incubated with miRNA probes composed of locked nucleic acids (LNA; Exiqon) at 57°C overnight. Oligonucleotides including LNA have increased thermal stability and improved discriminative power with respect to their nucleic acid targets and are therefore specifically suited for the detection of low-abundant miRNAs (Vester and Wengel, 2004). LNA probes for miR-181a-1*, 532, 379, and 16 were 3'-labeled with a digoxigenin tail using the DIG Oligonucleotide Tailing Kit (2nd Generation; Roche Applied Science). Scrambled LNA probes were used as a negative control. Hybridized probes were detected using a peroxidase-coupled sheep anti-digoxigenin antibody (Roche Applied Science) and Cy3-coupled tyramide signal amplification TSA PLUS Fluorescence Kits (Perkin Elmer). Simultaneous immunostainings were conducted using a rabbit MAP2 antibody (Sigma) and a mouse Tau antibody (Sigma), followed by Cy5- and Cy2-coupled anti-rabbit and anti-mouse secondary antibodies, respectively. FISH images were visualized using a 40X/1.3 Plan Fluor objective and a metal halide lamp (Nikon Intensilight C-HGFIE) on a Nikon Eclipse Ti inverted microscope. Images were captured with a cooled CCD camera (Cool-SNAP HQ, Photometrics) using NIS Elements (version

3.22.14, Nikon). Images were deconvolved using AutoQuant X2 (Media Cybernetics) and further processed using ImageJ (NIH). Line-scan quantification of FISH signals was analyzed along straightened axons. Mean pixel intensities of a 20 pixel wide line spanning the entire axon from the cell body to the outer tip of the growth cone were quantified using Image J. Mean intensities of consecutive segments of 20 pixel length were plotted as a function of distance to the cell body.

Statistics

Statistical analysis for miRNA profiling was performed in RealTime StatMiner (Intergenomics). Ago2 immunoprecipitation experiments were analyzed using SPSS Statistics 20 (IBM). Data were tested for normality and homogeneity of variances, and appropriate tests were used as indicated for each figure.

Results

Profiling of miRNAs from Distal Axonal Fraction

To identify specific miRNAs localized in axons by a biochemical approach, it is necessary to limit contamination of axons by miRNAs from the cell bodies. In *in vitro* cell culture preparations, this can be achieved by collecting only distal portions of long-extended axons to limit the presence of miRNAs that have entered axons by passive diffusion. For this purpose, we developed a novel culture method, termed “neuron ball” culture (Sasaki et al., 2010). Neuron balls prepared from mouse cortex extend axons up to 1–2 mm for 5 days after placing neuron balls on PLL-coated dishes, whereas dendrites extend only 20% of the length of axons, as visualized by Tau and MAP2 staining, respectively [Fig. 1(A,B)]. We used AraC to kill dividing cells to minimize content of glial cells in neuron ball culture, and cultures were visually checked for the absence of glia cells after the treatment. To purify axonal fractions, we removed cell bodies and associated proximal axons and MAP2-positive neurites by suction using sawed-off pipette tips (about 1 mm diameter). We collected distal axons that were about 500 μ m away from cell bodies, thereby minimizing contamination with dendrites and possible influx of miRNAs by passive diffusion from cell bodies. Most of the remaining neurites were axons, as determined by immunostaining with markers for axons and dendrites [Fig. 1(B)]. Furthermore, contamination of axon fractions from the cell body was less than 0.1%, as judged by qRT-PCR of nucleolar RNA (see Materials and Methods section). These observations indicate that the neuron ball method is suitable to collect very pure axonal RNA. Typically, we recovered about 20–30 ng of total RNA from the remaining distal axonal fractions obtained from 1200 neuron balls (one neuron ball contains 10,000 cells). This amount of RNA from the axonal fractions corresponded to about 0.02% of total RNA recovered from cell body fractions. An equal amount (5 ng) of RNA both from axonal and cell body fractions was analyzed by TaqMan miRNA Assay, a qRT-PCR-based assay system using 384-well plates. Of the 375 miRNAs analyzed in this assay, 159 miRNAs were detected both in axon and cell body fractions. After omitting miRNAs with Ct values above the cutoff value (Ct > 33), we analyzed 105 paired Ct values of miRNAs (Supporting Information Table S1).

The histograms of the Ct values of miRNAs in axons and cell bodies show that both distributions can be approximated as normal distributions judged by a D'Agostino and Pearson test [Fig. 1(C)]. The mean Ct value of miRNAs in axonal fractions (Ct_Ax: 28.41 ± 0.29) was higher than that of miRNAs of cell body fractions (Ct_CB: 26.36 ± 0.31), suggesting that the majority of miRNAs is more abundant in cell bodies than axons (Supporting Information Table S1). Because there is no standard control miRNA known that is expressed equally in axons and cell bodies, which would be necessary for the Δ Ct method, we instead used the Δ Ct method to compare miRNAs in axon and cell body fractions, similarly as Kye et al. (2007) used to detect miRNAs in dendrites and cell bodies. The overall mean Δ Ct (Ax – CB) was 2.06 ± 0.17 , and scatter-plot analysis of mean Ct values of individual axon–cell body pairs of miRNAs revealed a positive correlation between Ct_Ax and Ct_CB [Pearson correlation = 0.991; Fig. 1(D)], suggesting that most of the miRNAs distribute with a nearly constant gradient from cell bodies to axons. A similar cell body-dendrite gradient of miRNAs was reported recently in hippocampal neurons after 15 days *in vitro* (Kye et al., 2007). However, some populations of miRNAs deviate from the cell body-axon gradient. For example, Δ Ct (Ax – CB) of 12 miRNAs were less than 0 (Table 1), indicating that these miRNAs are localized more in axons than cell bodies. Moreover, of these 12 miRNAs, six were significantly enriched in axons when compared with the mean cell body–axon gradient calculated from all miRNAs (Table 1). miR-134, which was reported to be enriched in synapses (Schratt et al., 2006), and detected in axonal growth cones by FISH (Han et al., 2011) was also enriched in axons in our preparation, although the Ct value of cell bodies was below our cutoff value (i.e., 33; Table 1). miRNAs with a mean Δ Ct (Ax – CB) of over 4 were defined as cell body-enriched miRNAs (Table 2).

Candidate miRNAs Are Associated with RISC *In Vivo*

Recently, several miRNA sequences were removed from the miRBase database of miRNA sequences, because experimental evidence suggested that these short RNAs are not “real miRNAs,” but fragments of tRNAs or RNase P RNA. To validate the candidate miRNAs of interest, we therefore examined whether these miRNAs expressed in cortical neuron balls were associated with the RISC in the cortex of adult mice (postnatal day 21), which would imply their function *in vivo*. We quantitatively analyzed the association of miRNAs with RISC in mouse cortex by immunoprecipitation using an Ago2-specific antibody followed by qRT-PCR (Fig. 2). miR-16, 181a-1*, and 532 were shown to associate with Ago2 by immunoprecipitation [Fig. 2(B)]. miR-379 was also pulled down with anti-Ago2 antibody, however with less efficiency than the above three miRNAs. miR-685, which is now considered as a fragment of RNase P and was recently removed from the miRBase database of miRNA sequences, was not associated with Ago2. We obtained similar results using hippocampal tissue from adult mice (Supporting Information Fig. S1A), suggesting that miRNA function may be conserved between brain regions. These results support the suitability of our neuron ball method to validate miRNAs involved in RNA-induced silencing *in vivo*.

FISH Using LNA Probes Detected miRNAs in Axons and Growth Cones

We used LNA probes for FISH analyses to validate the localization of the above axon-enriched miRNAs shown to be associated with the RISC *in vivo*. FISH signals of miR-181a-1* (passenger strand of miR-181a-1) and miR-532, two axon-enriched miRNAs (Table 1), distributed as distinct granules in axons and growth cones as well as cell bodies in cultured cortical neurons [Fig. 3(A)]. Line-scan analysis revealed that the density of the miRNA granules in the axons decreased gradually toward distal axons; however, the density appeared to increase again in peripheral axons and growth cones [Fig. 3(D)]. This distribution pattern in axons and growth cones suggests a specific mechanism of nonrandom localization of miRNAs in peripheral axons and growth cones. We observed a similar localization of miRNAs in axons and growth cones of dissociated hippocampal neurons (Supporting Information Fig. S1B–D), suggesting conservation of miRNA localization among different brain regions. On the other hand, miRNAs enriched in cell bodies, miR-16 and miR-379 (Table 2), were localized as granules predominantly in cell bodies and proximal, but rarely in distal axons [Fig. 3(B,D)]. The negative control probe, a scrambled miRNA sequence, showed only background signals in the cell body, axon, or growth cone [Fig. 3(C)]. These FISH data confirmed the qRT-PCR results from profiling neuron balls. These results further indicate that miRNAs enriched in axons can also be localized to growth cones as granules.

Discussion

The identification of miRNAs localized in distal axons and growth cones is an important first step to elucidate mechanisms of local protein synthesis regulation by miRNAs in distal axons and growth cones. We have purified miRNAs from distal axons of cortical neurons and identified six axon-enriched miRNAs among over 100 miRNAs present in axons. Our culture method, neuron ball culture, allowed us to collect pure axons from larger quantities of neurons when compared with other methods using special apparatus and equipment such as Campenot and microfluidic chambers (Taylor et al., 2009; Natera-Naranjo et al., 2010) and laser capture microdissection (Kye et al., 2007). Compared to only 250–30,000 neurites that were collected by laser capture microdissection for miRNA and mRNA profiling (Kye et al., 2007; Zivraj et al., 2010), we were able to analyze axons derived from 1.2×10^7 neurons (10,000 neurons per one ball \times 1200). In general, larger quantities of samples result in more precise profiling. Furthermore, by using distal axons over 500 μm away from cell bodies, our protocol minimizes contamination with dendrites and possible influx of miRNAs highly expressed in cell bodies by passive diffusion. Our result detecting enrichment of miR-134 in distal axons is consistent with our recent findings that miR-134 is localized to axonal growth cones (Han et al., 2011). miRNAs enriched in cortical axons in the current study differ from those isolated in sympathetic axons (Natera-Naranjo et al., 2010), which may suggest differences in axonal miRNA profiles between neuron types and/or differences due to experimental or culture conditions.

Using FISH, we verified that miR-181a-1* and miR-532, identified as miRNAs enriched in distal axons in our screen, are localized to distal axons and growth cones. This specific localization in distal axons and growth cones is unlikely due to passive diffusion of miRNAs

with higher expression in cell bodies, because FISH analysis indicated that miR-181a-1* and 532 granules in growth cones were more abundant than those in the middle portion of axons, and qRT-PCR analysis revealed that miR-181a-1* and 532 content in distal axons over 500 μm away from cell bodies were more abundant than that in cell bodies. In contrast, although miR-16 and 379, which are cell body-enriched miRNAs according to our screen, are more abundantly expressed in cell bodies than miR-181a-1* and 532 as shown by qRT-PCR analysis (Tables 1 and 2), they were rarely detected in distal axons and growth cones. These two cell body-enriched miRNAs are probably spreading into axons with passive diffusion. In contrast, the axonal miRNAs, miR-181a-1* and 532, were clearly localized and seemed to be enriched in growth cones when compared with the cell body-enriched miR-16 and 379, suggesting the possibility of a mechanism to localize specific miRNAs into distal axons. Localized miRNAs in distal axonal compartments and growth cones may regulate local protein synthesis necessary for axon growth and guidance. This hypothesis is supported by the detection of miR-134 in axonal growth cones of *Xenopus* neurons and the identification of a role for miR-134 in translation-dependent growth cone guidance (Han et al., 2011). We identified miR-134 as one of the miRNAs enriched in axons (Table 1). Therefore, it is possible that miR-181a-1* and 532 may similarly play important roles in axon growth and guidance.

It will be interesting to investigate the localization of the axon-enriched miRNAs, which we identified in developing cultures, in developing axons *in vivo*. In addition, it will be of interest to identify miRNAs localized in mature myelinated axons *in vivo*. As mRNA expression in axons decreases in older neurons *in vitro* (Kleiman et al., 1994), localization of miRNAs in mature axons may be similarly reduced *in vivo*, which might make their detection more difficult. To ensure detectable amounts of miRNA for the current study, we therefore used developing axons of embryonic cortical neurons for the analysis of miRNA localization. It would be of interest if miRNAs may possibly be exchanged between oligodendrocytes and neurons by exosomes (Fruhbeis et al., 2012). Owing to the role of Dicer in axon regeneration *in vivo* (Wu et al., 2012), future work may uncover localization of specific miRNAs that play functional roles. Our current data on axon-enriched miRNAs in developing neuronal cultures will encourage future studies to examine the localization of miRNAs in axons *in vivo*.

In conclusion, we have identified several novel axon-enriched miRNAs and validated RISC-association and growth cone localization for two of these miRNAs. We emphasize that lack of evidence for axon-enrichment does not imply lack of axonal function. In our analysis, most of the miRNAs were not enriched in axons; however, the majority of the miRNAs were present in axons, and some were even quite abundant, although not enriched. This includes, for example, miR-9 and miR-19a, which were recently shown to be present in axons and growth cones of cultured cortical neurons (Dajas-Bailador et al., 2012; Zhang et al., 2013). These studies suggested that miR-9- and miR-19a-mediated regulation of the axonal synthesis of Map1b and PTEN are important for axon growth; however, the mechanism of localization or function of miR-9 and 19a in growth cones is not known. In our screen, both miRNAs (miR-9 and 19a) are prevalent in both axons and cell bodies, but are not enriched (Supporting Information Table S1). The axon-enriched miRNAs identified

here, which distribute nonuniformly in axons and are localized to growth cones, yet are present at lower levels in the soma, will be ideal candidates to characterize mechanisms that regulate miRNA transport and miRNA-mediated local protein synthesis in growth cones in response to extracellular signals. Major questions are, for example, whether miRNAs are cotransported with target mRNAs into axons, and how spatiotemporal events of miRNA-mediated target mRNA repression and derepression are regulated by extracellular signals. We anticipate that the axon-enriched miRNAs localized to growth cones will be shown to regulate translation of mRNAs encoding proteins with important axonal functions. These future studies will provide new insight into the mechanisms of local protein synthesis regulation and their function in axon growth and guidance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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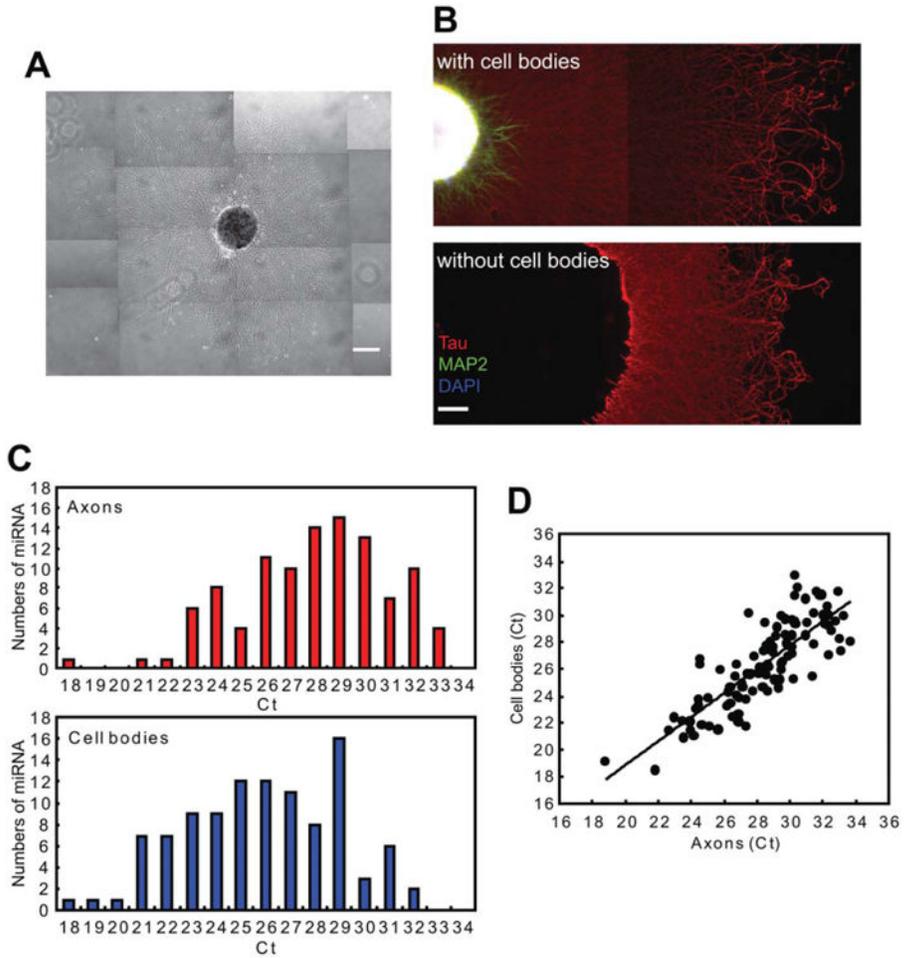


Figure 1. Profiling of axonal miRNAs. **A:** A phase-contrast image of the mouse neuron ball culture containing 10,000 cells. Scale bar = 200 μm . **B:** Neuron balls were stained by antibodies against Tau (axonal marker, red) and microtubule associated protein 2 (MAP2, dendritic marker, green). (Upper) Single, intact neuron ball. (Lower) Axons after removal of cell bodies by suction. Note that no MAP2 is detectable after removal of the cell body and surrounding neurites. DAPI (blue) was used for identification of nuclei. Scale bar = 100 μm . **C:** Histogram of Ct values of miRNAs quantified by real-time RT-PCR. RNA was purified from axonal and cell body fractions of mouse cortical neuron balls, and miRNAs were quantified by real-time RT-PCR using 375 primers specific for mature miRNAs. Ct values are represented by means of three individual experiments. **D:** Scatter-plot analysis of mean Ct values of individual axon-cell body pairs of miRNA. Logarithmic plots of the Ct values of axons and cell bodies are presented as relative expression values.

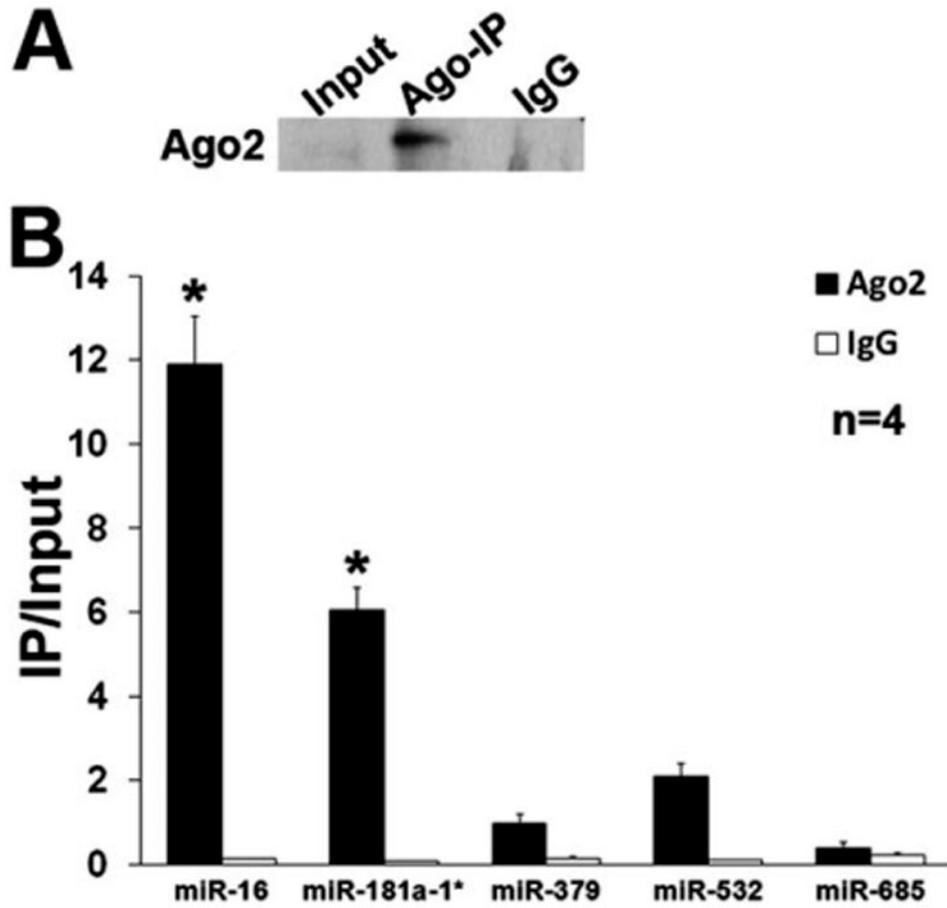


Figure 2.

RISC association of select candidate miRNAs. miRNAs were validated by verifying their presence in the RISC. Association of miRNAs with RISC was assessed by miRNA-specific qRT-PCR on RNA isolated from Ago2-immunoprecipitates from mouse cortex and hippocampus (see Supporting Information Fig. S1A). Immunoprecipitation with mouse IgG served as negative control. A: Western blot showing specific precipitation of Ago2 from hippocampal lysates with the Ago2-antibody, but not with IgG. B: Levels of immunoprecipitated miRNAs in cortical lysates were quantified by qRT-PCR and normalized to input levels. miR-16, -181a-1*, -379, and -532 are enriched in Ago2-immunoprecipitates when compared with IgG-immunoprecipitates. In contrast, “miR-685,” which has been identified as a false positive and was recently removed from the miRBase database of miRNA sequences, was not enriched ($n = 4$; two-way ANOVA; $*p(\text{Ago2 vs. IgG}) < 0.001$, $*p(\text{miRNAs}) < 0.001$, $*p(\text{interaction effect of miRNAs versus IP}) < 0.001$; LSD post hoc analyses $*p < 0.05$). Error bars represent SEM.

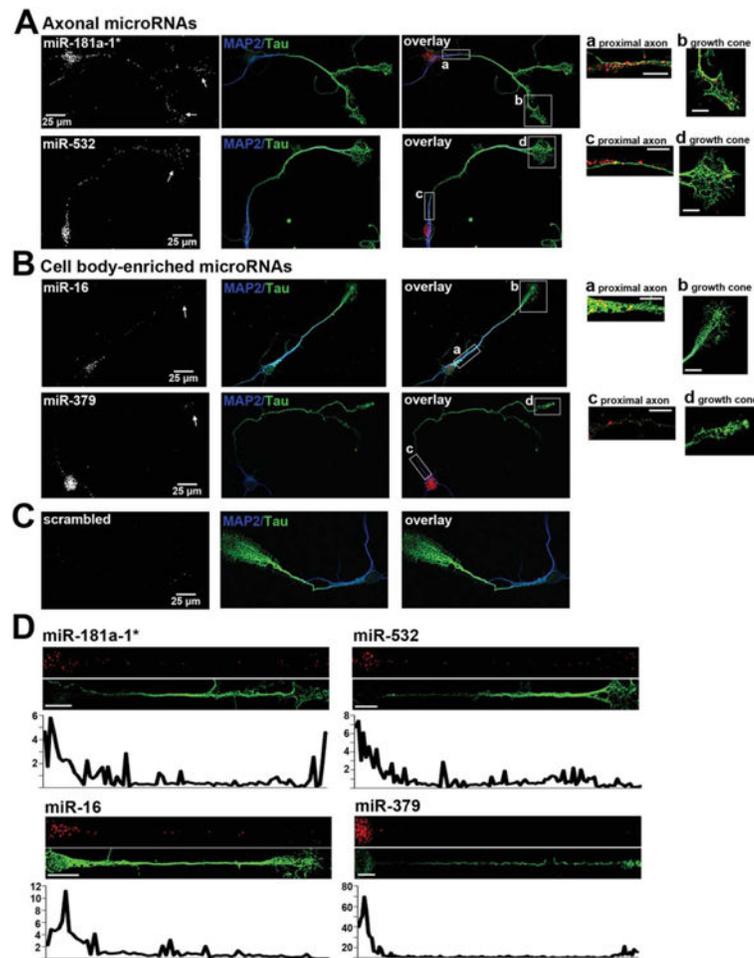


Figure 3.

Validation of axon- and cell body-enriched miRNA candidates by FISH. A: FISH analyses using digoxigenin-labeled LNA probes specific for miR-181a-1* and miR-532 (enriched in axons) show specific signal in axons and growth cones of cultured cortical neurons at 4 days *in vitro*. MAP2 (blue)- and Tau (green)-specific immunostainings were used to identify axons and dendrites. Arrows indicate growth cones, and scale bars in lower magnification images are 25 μm . Higher magnification images of FISH signal and Tau staining in proximal axons (a and c) and growth cones (b and d) (as indicated by white boxes) are shown for each example; scale bars are 10 μm . B: In contrast, FISH signals for miR-16 and miR-379 (enriched in cell bodies) are mainly restricted to cell bodies and proximal neurites. Higher magnification images of FISH signal and Tau staining (a-d) are indicated as in (A). C: A scrambled control only shows background signal in all cell compartments. D: Line-scan quantification of straightened axons for each example illustrates enrichment of miR-181a-1* and miR-532 in growth cones and distal axons, whereas miR-16 and miR-379 are mainly located to the cell body. Line scan was performed using Image J as described in the “Materials and Methods” section. Scale bars are 25 μm . Note that axons were scaled to the same length for each example.

Table 1

Axon-Enriched miRNAs

miRNA	Axons			Cell Bodies			Ct (Ax - CB)			Bonferroni
	Mean Ct	SE		Mean Ct	SE		Mean Ct	SE		
1 miR-361	30.28	0.17		32.90	0.79		-2.63	0.86		Significant
2 miR-532	27.55	0.47		30.13	0.72		-2.59	0.26		Significant
3 miR-685	24.53	0.63		26.72	0.33		-2.19	0.31		Significant
4 miR-720	24.56	0.32		26.31	0.42		-1.75	0.49		Significant
5 miR-181a-1*	30.47	0.23		32.01	0.55		-1.53	0.74		Significant
6 miR-17-5p	30.29	0.31		31.42	0.89		-1.13	0.58		
7 miR-676	28.54	0.26		29.43	0.89		-0.89	0.89		
8 miR-192	29.52	1.20		29.91	0.59		-0.40	1.45		
9 miR-709	18.76	0.12		19.13	0.19		-0.37	0.07		Significant
10 miR-324-3p	30.96	0.39		31.17	0.68		-0.21	0.91		
11 miR-382	25.78	0.34		25.91	0.60		-0.12	0.93		
12 miR-212	31.68	1.60		31.73	0.71		-0.05	2.07		
miR-134 ^a	30.45	0.93		35.12	1.44		-5.54	0.63		

Ax, axons; CB, cell bodies.

Data were obtained from three biological replicates. Significant: $p < 0.05$ (Bonferroni).

^a miR-134 is not ranked, because Ct_CB was too high (>33, see text in detail).

Table 2

Cell Body-Enriched miRNAs

miRNA	Axons		Cell Bodies		Ct (Ax - CB)		Bonferroni
	Mean Ct	SE	Mean Ct	SE	Mean Ct	SE	
105 miR-379	31.41	1.00	25.42	0.20	5.99	1.18	Significant
104 miR-188	33.12	1.30	27.33	0.16	5.79	1.18	
103 miR-423	33.68	0.77	28.01	0.06	5.67	0.71	Significant
102 miR-328	27.35	0.64	21.70	0.26	5.65	0.63	Significant
101 miR-135a	32.42	0.46	27.09	0.11	5.33	0.45	Significant
100 miR-195	30.27	0.61	25.25	0.10	5.01	0.52	Significant
99 miR-218	26.88	0.46	22.13	0.27	4.75	0.21	Significant
98 miR-124a	33.03	0.84	28.28	0.20	4.75	0.74	
97 miR-296	29.28	0.38	24.56	0.25	4.72	0.24	Significant
96 miR-16	28.70	0.48	24.36	0.07	4.34	0.41	
95 miR-434-3p	26.93	0.45	22.59	0.11	4.34	0.37	Significant
94 let-7g	29.41	0.68	25.20	0.19	4.21	0.87	
93 miR-9*	25.64	0.19	21.50	0.13	4.14	0.20	Significant
92 miR-137	26.54	0.30	22.49	0.05	4.05	0.34	Significant

Ax, axons; CB, cell bodies.

Data were obtained from three biological replicates. Significant: $p < 0.05$ (Bonferroni).