Supplemental Figure 1.

Northern blot analysis of let-7 expression in mouse brain development.

Panel A: Early embryonic brain development. Total RNA was isolated from neural tissue representing embryonic days 12 to 17 (Lanes 1-6) and post-natal day 0 (Lane 7). Precursor RNAs are marked with an arrow. The gel was stained with ethidium bromide prior to transfer. The 5S RNA band is shown in the last panel as control for equal loading. Expression of each let-7 miRNA was low until E14, increased until E17 and leveled off thereafter. We did not examine time points before E12, nevertheless, these results suggest that the precursor forms may contribute significantly to in situ hybridization signals at earlier time points.

Panel B: Early postnatal development. RNA from the cerebellum (B) was prepared separately from the remaining tissue, including midbrain, basal ganglia and cerebral cortex (denoted C) as indicated above each lane. Developmental stage for each pair is indicated (P2, P5, P8 and P14). Images were cropped to show all specific signals obtained with each probe.

Panel C: Expression of primary, nuclear let-7 miRNA transcripts in mouse brain development. RT-PCR was performed on total RNA from selected time points from E13 to P14, indicated above each lane. Primary transcripts were amplified with primers specific for let-7a-1/let-7f-1 (pri-let-7a) or mir-99/let-7e/mir-125a (pri-let-7e) (Lanes 1-5). Control reactions were performed on mock cDNA prepared without addition of reverse transcriptase (RT) (Lanes 6-10). cDNA integrity was confirmed using primers specific for β-actin.
A. 

B. 

C. 

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- pri-let-7a
- pri-let-7e
- β-actin
Supplemental Figure 2

A let-7 sensor construct is differentially regulated in astrocytes and neurons.

**Panel A:** Quantitative analysis of eGFP expression in EC cells stably transfected with a let-7 sensor derived from the C. elegans *lin-41* gene in peGFP-lin41S; the antisense control plasmid peGFP-lin41AS; the point mutant peGFP-lin41mut or the vector peGFP-N1 as indicated in the legend. The proportion of eGFP* cells as determined by flow cytometry prior to neural induction was set at 100% and compared to eGFP* cells twelve days after stimulation with RA. Data are from five independent experiments (p<0.001, Student’s paired t-test).

**Panel B:** Efficient downregulation of the let-7 sensor in EC-derived neurons. Representative plates of sensor and control transfectants prior to (-RA), and 12 days after stimulation with RA (+RA). For differentiated cultures, neuronal phenotype is demonstrated by staining with the Tuj1 antibody.

**Panel C:** Comparison of let-7 sensor expression after transfection in embryonic astrocytes and neurons. GFP expression was quantified by flow cytometry and expressed for each plasmid tested relative to the parent vector peGFP-N1. Values were normalized for transfection efficiency monitored by co-transfection with pDsRed2-N1. Representative flow cytometry plots are presented in Supplemental Figure 3. Statistical significance for the reduction in sensor expression in each set of experiments was confirmed using the t-test (n=4, p<0.01).

In astrocyte transfections the proportion of GFP-expressing cells was essentially equivalent for peGFP-N1, the antisense, and the mutant control plasmids. eGFP
expression mediated by the let-7 sensor plasmid was modestly suppressed (60% of control), consistent with low levels of let-7 activity in astrocytes.

Quantification of eGFP-expressing cells by flow cytometry after electroporation of E15 neuronal cultures revealed very similar levels for the antisense and point mutant compared to the parent vector. The let-7 sensor, however, was strongly downregulated: eGFP-expressing cells were reduced to 20% of control. Average fluorescence intensity was more strongly affected than the tally of eGFP⁺-positive cells, as is apparent from the corresponding flow cytometry plots (see Supplemental Figure 3). Neuronal identity of the overwhelming majority of transfected cells was confirmed by co-staining with neuron-specific β-III tubulin antibody (data not shown, see also Materials and Methods).
A

% GFP-Positive

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B

Sensor

Control

eGFP - RA

eGFP + RA

C

% GFP-Positive

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Supplemental Figure 3

Representative flow cytometry plots of eGFP expression (GFP Fl-1) versus side scatter (SSC). Gates were established with untransfected cells, for each population the percentage of positive-gated cells is given. In Panel A, transfections of primary cortical astrocytes with peGFP-lin41S (Sensor), peGFP-lin41AS (Control) and peGFP-lin41mut (Mutant) are shown. Panel B displays results obtained by electroporation of primary cortical neurons. In Panel C EC cell populations stably transfected with each construct were assayed prior to and 12 days after induction of neural differentiation, as indicated.
Supplemental Figure 3

A  Astrocytes

B  Neurons

C  Undifferentiated EC  Neural-Induced EC
Supplemental Figure 4

Panel A and B: Semi-quantitative RT-PCR analysis of miRNP components. cDNA samples were prepared from mouse embryonic stem cells (ES), P19 embryocarcinoma cells (EC), and primary (Pri.) embryonic astrocytes (A) and neurons (N). ES samples were taken from undifferentiated cells (U); cells stimulated with RA and cultured for 24 days (N⁺); cells undergoing neural differentiation in defined medium (N⁻); or ES cell-derived cardiomyocytes (C). EC samples were from undifferentiated cells (U) or cells stimulated with RA and cultured for 12 days (N⁺). Genes targeted by primer pairs are given on the left. Note the increased signal for FMRP in Panel B, Lanes 2 and 4.

Panel C: Immunohistochemistry of RA-treated EC cells revealing high-level FMRP expression (green) in Map-2 positive cells (red). Arrows mark representative double-positive cells.

Panel D: FMRP and marker expression of neuron and astrocyte cell extracts. Western blots of cytoplasmic extracts from astrocytes (A) and neurons (N), as indicated to the right of each lane, were probed with antibodies against FMRP (clone 1C3), neuron specific β-III tubulin, GFAP and β-actin. Similar results were obtained with whole cell extracts (data not shown).

Panel E: Comparison of miRNA precursor binding complexes formed on pre-let-7a with extracts from primary embryonic astrocytes (A, Lane 1) or neurons (Lane 2). Two prominent complexes are seen using neuronal extracts, labeled A and B to the right. Complex B formation is enhanced in neuronal extracts compared to astrocytes.
Supplemental Figure 4

A

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Dicer

Gemin3

Gemin4

Ago1

B

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FMRP

β-Actin

C

FMRP

Map-2

D

E

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FMRP

β-III tubulin

GFAP

β-Actin

MOV10

TRBP2

TNRC6B
Supplemental Figure 5

Panel A: Western blot comparing Ago1 expression before (U, Lane 1) and after differentiation (N, Lane 2) of EC cells. The position of Ago1 is marked by an arrow to the right.

Panel B: Western blot displaying specificity of the Ago1 antibody used. Extracts from non-transfected HEK293 cells (Lane 1) and cells transfected with Ago2 (Lane 2) or Ago1 (Lane 3). The position of Ago1 is marked by an arrow to the right, note detection of overexpressed Ago1 but not Ago2.

Panel C: Endogenous Ago1 protein was visualized in HEK293 and EC cells with anti-Ago1 antibody, as indicated above each panel. Prominent cytoplasmic foci are evident in HEK293 cells but not EC cells.
A. P19

B. HEK293

Ago1: - - +
Ago2: - + -

C. HEK293

P19 EC

Ago1
**Supplemental Figure 6.** Localization of miRNA processing proteins in cultured hippocampal neurons. **Panel A:** Neurons were co-transfected with expression vectors for Flag-tagged Ago1 and c-myc-tagged MOV10. Ago1 was visualized with anti-Flag mAb M2 (green), MOV10 with rabbit anti-myc (red). The top panels provide an overview of a positive neuron, the lower panels a detailed view of dendritic staining. **Panel B:** Neurons were co-transfected with expression vectors for Flag-tagged FMRP and c-myc tagged MOV10. FMRP was visualized with anti-FMRP mAb 1C3 (red), MOV10 with rabbit anti-myc (green). An overview and detailed view of dendritic staining are provided as above. **Panel C:** Detailed view of dendritic staining of a neuron co-transfected with expression vectors for eGFP and c-myc tagged MOV10. Cells were stained for eGFP (anti-eGFP mAb, green) and MOV10 (rabbit anti-myc, red). **Panel D:** Flag-tagged Ago1 and c-myc-tagged TNRC6B were co-transfected and visualized with anti-Flag mAb M2 (green), and rabbit anti-myc (red), respectively. Staining was confined to foci in the neuronal soma and proximal dendritic arbour, three examples are marked with arrows.
SUPPLEMENTAL METHODS

in situ hybridization

LNA probes (Exiqon) were labelled with terminal transferase and digoxigenin-ddUTP (DIG-3'-end labelling kit, Roche) for whole mounts or 35S-labelled dATP for cryosections followed by purification with MicroSpin G-25 columns (Amersham Biosciences). E9.5 embryos were treated with Proteinase K (20µg/ml) at RT for 7 min, rinsed in PBST and fixed in 4% PFA for 20 min at RT. Embryos were washed three times with PBST, rinsed twice with pre-warmed hybridization solution and prehybridized at 20°-22°C below the calculated probe Tm. After 2h the prehybridization mix was replaced with 1 ml of hybridization buffer (HB) containing 10 µl of purified probe and hybridized overnight. 15 min wash steps at hybridization temperature followed in HB, 50% HB/50% 2 x SSC, 2 x SSC, and 0.2 x SSC at hybridization temperature for 15 min each. Further wash steps in 50% 0.2 x SSC/50% PBST, and PBST were performed at RT followed by blocking in PBST plus 2% sheep serum and 2 mg/ml BSA. Incubation with anti-DIG antibody (1:5000) was overnight at 4°C. Embryos were washed 6 x with PBST for one hour each, then 2 x in NTMT (100mM Tris-HCL pH 9.5, 50mM MgCl2, 100mM NaCL, 0,1% Tween) for 15 min and stained with BCIP/NBT in NTMT. For sections, embryos were embedded in gelatine/PBS and fixed overnight in 4% PFA. Whole mounts and 50µm vibratome sections were photographed with a LEICA MZ-16FA stereomicroscope.

Pre and postnatal cryosections were prepared using standard procedures, and hybridized at 55°C with 500,000 cpm of probe in 50 µl of hybridization buffer (10 mM HEPES pH 7.5, 600 mM NaCl, 100 mM dithiothreitol, 50%(v:v) formamide, 1mM EDTA, 10% dextran sulfate, 1x Denhardt’s solution, 100µg/ml salmon sperm DNA, 100µg/ml
yeast tRNA) overnight. Sections were washed four times in 1xSSC at 65°C for 15 min, dehydrated, air dried, and exposed to X-ray film (Kodak) for 3-5 days.

**Northern Blotting and RNA Isolation**

For the isolation of RNA from mouse embryos and neonates of timed pairings, brain tissue was dissected, meninges removed, followed by collection in liquid N₂. When indicated in the text, the cerebellum was dissected prior to freezing. Total RNA was isolated from mouse tissue and cell lines using the Trizol reagent following the manufacturer’s recommendations.

**Cell Culture**

Neural induction of P19 EC and D3 ES cells has been described in detail (1). Briefly, 1 x 10^6 EC cells were stimulated two times with 500 nM all-trans retinoic acid over four days and allowed to aggregate. Resultant embryoid bodies (EB) were plated in α-MEM plus 10% FCS. After a further three days, medium was changed to Neurobasal medium plus B27 (GIBCO).

For cardiomyocyte differentiation of ES cells, hanging drop cultures were incubated for three days without mLIF and then transferred to bacterial petri dishes for another two days. On Day 5, individual EBs were transferred to a 24-well tissue culture plate. >95% of colonies displayed pacemaker contractions at Day 11. For neural differentiation, cells cultured as above were incubated with 500 nM retinoic acid from Day 3 to Day 5. Differentiation was monitored by flow cytometry using antibodies against Internexin, Map-2 and GFAP.

Primary cortical neurons and astrocyte cultures were prepared as described in (1). Mouse hippocampal neuron preparation and culture is described in (2).
**Plasmids**

The *lin-41* 3′UTR containing *let-7* complementary sites (LCS) were amplified from the plasmid pEYC1 (3) using the following primers:

5′ GCGGCCGCGGATCCCGCAGTGAAATTTGCGA 3′ and 5′ GCGGCCGCGAAATCTCAGGAAAAGTC 3′. Direct interaction of the *let-7* miRNA with at least two binding sites on the *lin-41* fragment is well established (4). PCR fragments were cloned into pCR2.1 TOPO (Invitrogen), followed by excision with NotI and insertion into the NotI site of plasmid peGFP-N1 (Clontech) situated within the 3′UTR of the eGFP mRNA to generate peGFP-lin41S and peGFP-lin41AS.

Mutations were introduced into peGFP plasmids using the QuickChange II Mutagenesis kit (Stratagene). For *lin-41* LCS1 was changed from 5′ TTATACAACCGTTCTACACTCA to 5′ TTATACAACCGTTCTACAGTC 3′, for LCS2 from 5′ TTATACAACCGATTCTGC 3′ to 5′ TTATACAACCGATTCTGCAGC 3′ (Primer sequences used are available upon request). Ago1, Ago2, MOV10 and TNRC6B expression plasmids pIRESneo-FLAG/HA Ago1, pIRESneo-FLAG/HA Ago2, pmyc-MOV10 and pmyc-TNRC6B were made available by T. Tuschl and are described in (5;6). The epitope-tagged FMRP construct was a gift of Y. Feng and is described in (7).

**RT-PCR**

Total RNA described above was treated with RNase free DNAse (Ambion), primed with oligo-dT and cDNA synthesized using Superscript III reverse transcriptase (Invitrogen) following manufacturer’s specifications. For each experiment, cDNA concentration was titrated using β-actin-specific primers in a standard PCR reaction at 20 to 25 cycles. Cycle number was optimized for each primer pair. Primers for amplification of the primary *let-7a-1/let-7f-1* transcript were derived from sequences flanking both precursors.
encoded on est’s (TTCACCCTGGATGTTCTCTTCA and CAGGGTAAATCATCCTACCCACA). For let-7e the primers correspond to sequences from the flanking miRNA precursors mir-99 and mir-125a (CACCCGTAGAACCACCTTGCG and CACAGGTTAAAGGGTCTCAGGA).

These amplicons were used for the design of primers and probes for Real-Time PCR (Applied Biosystems). Assays were performed by the TaqMan method on an ABI 7500 station. All amplifications were performed in duplicate, similar results were obtained using either 18S or GAPDH probes as standards. Assays were performed twice, using three independent cDNAs per cell line and differentiation condition. Further details are provided in (8).

**Primary Cell Transfection**

To generate stable clones EC cells were transfected with FuGENE6 (Roche) following manufacturer’s specifications and selected with 800µg/ml G418. Resistant clones were pooled and selected for eGFP expression by FACS.

Culture conditions and transfection of E15 astrocyte cultures with FuGene6 has been described (1). Astrocytes were transfected in duplicate with the sensor and control constructs in six well plates with FuGENE6. Transfection efficiency was monitored by co-transfection with pDsRed2-N1. eGFP and dsRed expression was quantified by flow cytometry relative to control peGFP-N1 transfections. Gates were established with control cells stained with anti-GFAP antibody (~85%).

For electroporation of primary cortical neurons, the Amaxa Mouse Nucleofector kit was used following manufacturer’s recommendations. Transfection efficiency was monitored by co-transfection with pDsRed2-N1; quantification was by flow cytometry as described
(1). For immunohistochemistry, hippocampal neurons were transfected with Effectene (Qiagen) after 10 days in culture as described (2).

Reference List


