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Expression of microRNAs and their precursors in synaptic fractions of adult mouse forebrain

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Abstract

We have characterized the expression of microRNAs and selected microRNA precursors within several synaptic fractions of adult mouse forebrain, including synaptoneurosomes, synaptosomes and isolated post-synaptic densities (PSDs), using methods of microRNA microarray, real time qRT-PCR, Northern blotting and immunopurification using anti-PSD95 antibody. The majority of brain microRNAs (especially microRNAs known to be expressed in pyramidal neurons) are detectably expressed in synaptic fractions, and a subset of microRNAs is significantly enriched in synaptic fractions relative to total forebrain homogenate. MicroRNA precursors are also detectable in synaptic fractions at levels that are comparable to whole tissue. Whereas mature microRNAs are predominantly associated with soluble components of the synaptic fractions, microRNA precursors are predominantly associated with PSDs. For seven microRNAs examined, there was a significant correlation between the relative synaptic enrichment of the precursor and the relative synaptic enrichment of the corresponding mature microRNA. These findings support the proposal that microRNAs are formed, at least in part, via processing of microRNA precursors locally within dendritic spines. Dicer is expressed in PSDs but is enzymatically inactive until conditions that activate calpain cause its liberation; thus, we propose that synaptic stimulation may lead to local processing of microRNA precursors in proximity to the synapse.

Keywords: dicer, fragile X mental retardation protein, microRNA, microRNA precursors, post-synaptic density, synaptic plasticity.

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Since the pioneering report of Lagos–Quintana *et al.* (2002), it is known that hundreds of microRNAs are expressed in mammalian brain, and there is strong evidence that they regulate protein translation both during development and in the adult (reviewed in Tai and Schuman 2006; Kosik, 2006; Fiore *et al.*, 2008). Furthermore, numerous components of the microRNA machinery, including dicer and the core RISC component eIF2c (an Argonaute homologue), are expressed within dendrites and are, in part, associated with post-synaptic densities (PSDs) (Lugli *et al.* 2005). Similarly, not only have polyribosomes and a broad range of mRNAs been detected within dendrites, but polyribosomes have been shown to be expressed within dendritic spines (especially after stimuli that elicit long-term potentiation) (Ostroff *et al.* 2002; Bourne *et al.* 2007) and mRNAs and translational regulatory components have been detected in proximity to PSDs as well (Asaki *et al.* 2003; Suzuki *et al.* 2007). Cytoplasmic bodies such as RNA granules, stress bodies and P bodies have also been identified within dendrites (Vessey *et al.* 2006; Ferrari *et al.* 2007).

A large body of evidence indicates that synaptic plasticity, dendritic spine growth, and learning are critically dependent upon regulation of specific protein synthesis near or within dendritic spines, and emerging evidence suggests that microRNAs play roles in these processes. For example, Schratt *et al.* (2006) showed in mouse that a specific microRNA, mir-134, regulates a specific dendritic mRNA, LIMK1, which is implicated in growth of dendritic spines. In *Drosophila*, Ashraf *et al.* (2006) showed that Armitage protein, a component of the RISC complex, was modified by proteolysis during long-term olfactory learning, and that learning was impaired in an *armitage* mutant. Fragile X mental retardation protein (FMRP), which regulates protein synthesis in dendritic spines and is critical for normal cognition, behavior, and spine development (reviewed in

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Abbreviations used: FMRP, fragile X mental retardation protein; HB, homogenization buffer; PSDs, post-synaptic densities; SYN, synaptoneurosomes; TBE, Tris-borate-EDTA.

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Vanderklish and Edelman, 2005) has recently been shown to interact with multiple components of the microRNA machinery which, at least in part, appear to mediate the effects of FMRP (reviewed in Qurashi *et al.* 2007).

Despite these advances, it is not clear whether a diverse population of microRNAs is expressed in dendrites and/or in dendritic spines, and whether they are as abundant near synapses as within the cell body compartment. Nor is it understood how microRNAs arrive at the synaptic compartment(s). There are at least three possible ways that microRNAs may arrive at dendrites (Tai and Schuman 2006; Kosik, 2006; Fiore *et al.*, 2008): First, mature microRNAs may passively diffuse into dendrites, which seems to be the predominant mode within neurons that are still maturing (Kye *et al.* 2007). Second, mature microRNAs may be processed from precursors in the neuronal cell body and then actively transported to dendrites, either by themselves or by 'piggybacking' on their mRNA targets as the latter become transported to dendrites. Third, primary microRNA gene transcripts (pri-miRs) or small hairpin precursors (pre-miRs) may be actively transported to dendrites, and then processed to microRNAs locally. These scenarios are not mutually exclusive. However, because dicer, the Rnase III enzyme that cleaves precursors into mature microRNAs, is expressed within dendritic spines and is highly enriched at PSDs (Lugli *et al.* 2005), we hypothesized that local processing of pre-miRs occurs near synapses. At present, however, there is no published evidence that microRNA precursors are detectably expressed at all within dendrites or synaptic fractions of adult mammalian neurons.

Materials and methods

Subcellular fractionation

Two-month old male C57Bl/6 mice were employed in the present study. Each prep consisted of a pool of 3–4 forebrains (including cortex and hippocampus). Synaptoneurosomes, synaptosomes and isolated PSDs were prepared using standard methods (modified to preserve RNA integrity) and each prep was characterized to ensure that it had the expected enrichment of specific protein and RNA components, as previously described (Smalheiser and Collins 2000; Lugli *et al.* 2005). Forebrain was rapidly dissected, placed in RNAlater (Ambion, Austin, TX, USA) for the time of the dissection of 3–4 mice and each pool was immediately homogenized using a Dounce pestle in ice-cold homogenization buffer (HB) containing a cocktail of protease and Rnase inhibitors [50 mM Hepes, pH 7.5, 125 mM NaCl, 100 mM sucrose, 2 mM K acetate, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, 10 μg/mL leupeptin, 1 μg/mL pepstatin A, 2 μg/mL aprotinin, 160 U/mL Superase-In (Ambion), 160 U/mL Rnase-OUT (Invitrogen Life Technologies, Carlsbad, CA, USA)]. Synaptoneurosomes were prepared as in Lugli *et al.* (2005), except that the synaptoneurosomes were quickly pelleted and rinsed twice in 10–20 × volume of homogenization buffer containing all inhibitors prior to extracting RNA. Synaptosomes were prepared as in Smalheiser and Collins (2000) but sucrose was treated with RNAsecure (Ambion) as per the manufacturer's instructions before making the sucrose gradient, and the sucrose solution was supplemented with Rnase-OUT (Invitrogen) 320 U/mL.

RNA isolation

Total RNA was isolated with Trizol reagent (Invitrogen) according to manufacturer's directions with a few modifications to maximize yield of small RNAs. Glycoblue 20 μg (Ambion) was added to the RNA precipitation step which was allowed to proceed overnight at −20°C. The RNA pellet was spun down at 20 000 *g* for 25 min at 4°C; rinsed with 80% ethanol in DEPC-treated water (Invitrogen); resuspended and treated with RNAsecure (Ambion); and treated with Dnase I using DNA-free (Ambion). Each prep was characterized for purity by OD 260/280 ratio and for integrity by running on agarose gels.

Northern blotting

Synaptoneurosomes were lysed with 1% Nonidet P-40, and the pellet was spun-down at 20 000 *g* for 20 min and rinsed twice with HB buffer prior to preparing total RNA. The RNA was heated (100°C, 3 min) in loading buffer type II (Ambion) and loaded (3.8 μg RNA for miR-124a, 0.5 μg RNA for BC1) in a 10% Tris-borate-EDTA (TBE) UREA criterion gel (Bio-Rad, Hercules, CA, USA). The gel ran in 1X TBE buffer at 120 V for 1 h and the RNA was transferred in 0.5X TBE buffer to Hybond-N+ (Amersham Biosciences, Piscataway, NJ, USA) for 30 min at 20 V. Membranes were UV-cross-linked for 2 min and baked at 80°C for 45 min, incubated in ULTRAhyb hybridization buffer (Ambion) for 45 min at 68°C on a shaker, then biotin-linked oligonucleotide probes were added (BC1 probe at 0.1 nM and miR-124a at 0.3 nM) with incubation overnight at 42°C on a shaker. Membranes were rinsed and signals were detected using the BrightStar BioDetect kit (Ambion) with 1 min exposure to Hyperfilm ECL (Amersham Biosciences). Probe sequences were as follows: BC1: aaaggttgtgtgtgccagttaccttgtttt; mir-124a: tgGcaTtcAccGcgTgcCttAa (lower case is DNA, upper case is Locked Nucleic Acid; probes were modified with biotin at both 5′ and 3′ ends; probes were prepared by IDT DNA, Inc., Coralville, IA, USA).

RT-PCR and real time qRT-PCR measurements

See Supplementary material File 1 for a list of all primer sequences.

Housekeeping and synaptic RNAs (BC1, GAPDH, CAMK2A, ribosomal 18S RNA and U6)

Total RNA (0.5 μg) was reverse transcribed with short gene-specific primers with T_m around 38–42°C using Superscript II (Invitrogen) as per the manufacturer's protocol with a few modifications. Denaturation was performed at 80–90°C for 5 min, followed by 5 min at 33°C, 30 min at 36°C and 30 min at 42°C. Real-time quantitative PCR was performed on a Stratagene MX 3005P real-time PCR Instrument (Stratagene, La Jolla, CA, USA). Each sample was run in duplicate or triplicate. All products were confirmed and distinguished from primer dimers by examining melting curves and by running the PCR product on 3.5% agarose gels. As negative controls, parallel samples lacked reverse transcriptase, or lacked added RNA sample. The PCR mix, 20 μL, contained 10 μL of SYBER GREEN PCR master mix (Applied Biosystems, Foster City, CA, USA) 0.4 μM of

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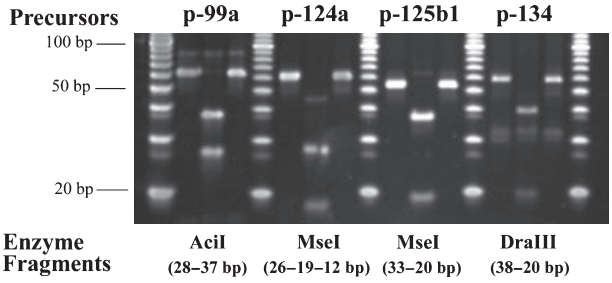

Fig. 1 Restriction enzyme digestion of qRT-PCR products for four microRNA precursors. PCR products were run (left lanes of each pre-miR as indicated) and gave the expected size appropriate to each microRNA precursor (pre-miR-99a = 62 bp; pre-miR-124a = 57–59 bp; pre-miR-125b1 = 53 bp; pre-miR-134 = 58 bp) relative to lanes loaded with 10 bp DNA ladder (Invitrogen). Products were also incubated with restriction enzymes (middle lane) or incubated without enzymes (right lane) as indicated. Enzymes (New England Biolabs) were added to 10 μL PCR product + 1.3 μL enzyme buffer +2 μL water and incubated at 37°C for 90 min, then resolved on a 15% TBE criterion gel (Bio-Rad) and stained with Syber Gold. Each restriction enzyme produced fragments of the expected sizes (as shown below the gel) appropriate to each microRNA precursor sequence.

each pair of primers, and 5 μL of dilute cDNA (1 : 50). PCR was performed for 20 s at 95°C, 25 s at 58°C and 25 s at 72°C for 40 cycles followed by the thermal denaturation protocol.

MicroRNA precursors

RNA (1 μg) was employed for each reaction. Primer pairs specific to each precursor were employed, following the protocol described in Jiang *et al.* (2005), employing mouse sequences. The PCR mix was as described above, and PCR was performed for 20 s at 95°C, and 50 s at 60°C for 50 cycles followed by the thermal denaturation protocol. In our hands, microRNA precursors were more 'finicky' to measure using RT-PCR than other types of RNAs, and for some pre-miRs (especially those forming relatively tight hairpins) it was necessary to modify the Schmittgen protocols by changing the primer sequences or by raising the denaturation temperature. (We were never able to analyze pre-miR-143 successfully, presumably because it forms a very tight hairpin structure.) Besides confirming PCR products by melting curves and by running the PCR products on agarose gels, selected pre-miRs were also tested to verify that they were cut by specific restriction enzymes giving pieces of the expected sizes (Fig. 1). Note that the PCR method does not distinguish between primary microRNA gene transcripts and microRNA precursors; thus, additional size selection experiments were carried out to distinguish pri-miRs from pre-miRs (see Results).

Mature microRNAs

Reverse transcription was performed in 20 ng of total RNA using specific TaqMan® MicroRNA Reverse Transcription and Assay kits (Applied Biosystems) following the manufacturer's protocol. MicroRNA specific PCR employed the TaqMan miRNA specific assay kit from Applied Biosystems. This recognizes dimeric PCR product in a sequence-specific manner and thus does not detect primer dimers. The looped primers are designed to avoid detecting pre-miRs. Although it is conceivable that some pre-miRs are recognized under some conditions (i.e., when the mature microRNA derives from the extreme end of the pre-miR), this would not have a practical effect on measuring mature microRNA levels because the steady-state levels of mature microRNAs are typically 20–50 times higher than that of the corresponding pre-miRs (e.g., Jiang *et al.* 2005; Schmittgen *et al.* 2008).

MicroRNA microarrays

Each microarray measured a single paired prep (total forebrain homogenate vs. synaptoneurosome fraction, SYN). Total RNA (10 μg) was size-selected and labeled with Alexa dye A3 (total homogenate) or A5 (SYN) by Invitrogen staff. The labeled RNA was mixed together and hybridized to a NCODE multi-species microRNA chip (Invitrogen version 1.0) using Maui hybridization. Invitrogen version 1.0 chips contain 2 spots for each annotated microRNA across multiple species – since many mouse, rat, and human microRNAs have identical sequences, up to six spots per array could be averaged for certain microRNAs. We removed from consideration any individual spots with high internal standard deviation (i.e., SD > 3X the median intensity value in either the red or green channel) as well as outlier spots (i.e., cases in which 1 or 2 spots had intensity values <2X background, and the remaining spots for that microRNA had intensity values > 3X background in either red or green channel). Raw intensity values were adjusted by subtracting local background values from each spot (any spot with resulting intensity <0 was assigned the value 1). The microarray intensity values, as well as the results of real-time RT-PCR measurements, indicate that the overall concentration of microRNAs relative to total RNA was similar in both the SYN fraction and the total homogenate (Fig. 2); the overall profile of intensity values was also similar across the three preps (data not shown). The intensity values were normalized further as described in Results.

Immunopurification with anti-PSD95 antibodies

Protein-G and Protein-A agarose beads were rinsed twice with HB buffer containing the cocktail of protease and Rnase inhibitors and once with HB diluted 1 : 1 with radioimmunoprecipitation (RIPA) buffer containing inhibitors. Purified synaptosomes (2.3 mg/mL) were lysed by diluting the suspension 1 : 1 with RIPA buffer (pre-treated with RNAsecure, Ambion and containing the protease and RNAase inhibitor cocktail) and pre-cleared with Protein-A agarose beads (Amersham) or with Protein-G agaorse beads (Amersham) for 60 min at 4C. Anti-PSD95 monoclonal antibody (University of California-Davis, clone 28/86, 1 mg/mL) or anti-synapsin I affinity purified polyclonal antibody (Chemicon, Temecula, CA, USA; AB1543P, 0.1 mg/mL) was added to pre-cleared synaptosomes and incubated over night at 4°C with gentle agitation. These were then mixed with protein-G beads (anti-PSD95 sample) or protein-A beads (anti-synapsin I sample) for 4 h, then the beads were rinsed twice with HB (diluted 1 : 1 with RIPA containing the inhibitor cocktail), once with HB, and once with 50 mM Tris pH 7.0. To analyze proteins associated with the immunoprecipitates, 40 μL Laemmli loading buffer was added, the beads were heated at 100°C for 5 min, and dithiothreitol was added (final concentration 1%). As negative controls, assays were conducted in the absence of primary antibodies and in the absence of synaptosomes. To analyze RNA

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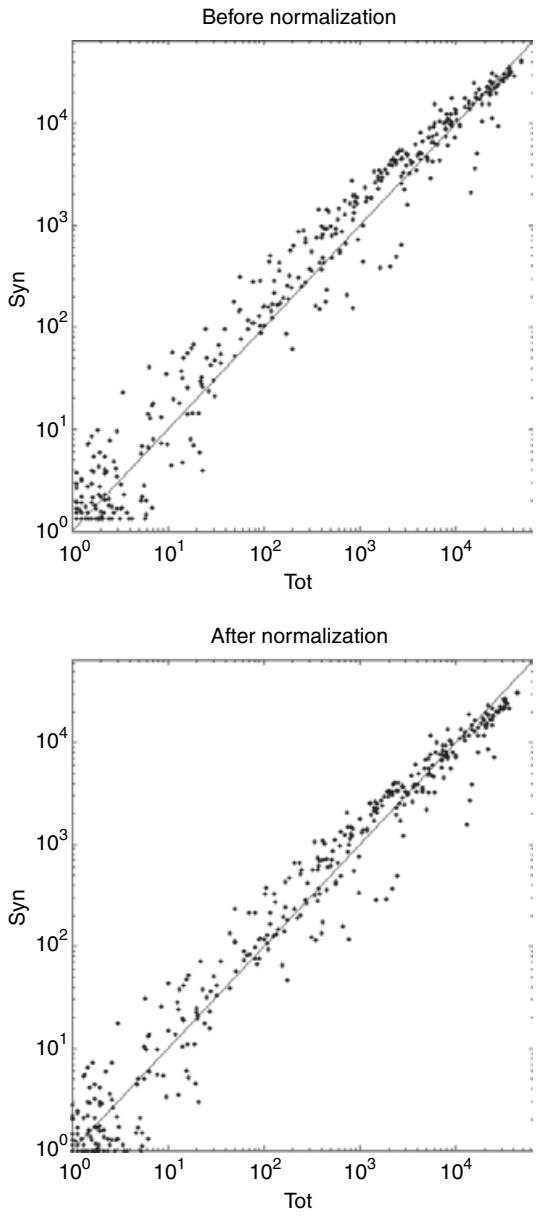

Fig. 2 microRNA intensity values in synaptoneurosomes versus total forebrain homogenate as measured by microarray. Data were plotted before versus after normalizing values to the mean value of the overall microRNA population as described in Results.

associated with the immunoprecipitates, Trizol 1 mL (Invitrogen) was added to rinsed beads and total RNA was extracted.

Western blotting

Western blotting was performed as in Lugli *et al.* (2005). Blots were blocked in 1% nonfat dry milk for 1 h, room temperature, incubated with primary antibody anti-PSD95 monoclonal antibody (Clontech, 1 : 30 000) or anti-Synapsin I affinity purified polyclonal antibody (Chemicon, AB1543P, 0.1 μg/μL) overnight with rocking at 4°C and rinsed; goat anti-mouse IgG (peroxidase conjugated; Chemicon) or goat anti-rabbit IgG (peroxidase conjugated; Sigma A0545) was added at 1 : 30 000–50 000 for 2 h and rinsed; finally, blots were incubated in ECL-Plus reagent (Amersham) and exposed to film (Hyperfilm ECL, Amersham).

Results

Synaptoneurosomes

Synaptoneurosomes are small vesicular structures that are prepared by filtration of total forebrain homogenate through mesh of progressively decreasing size, followed by low-speed centrifugation (discarding the pellet) and higher-speed centrifugation (keeping the pellet). This fraction is thought to be enriched in dendritic spines. They were prepared by a modification of Weiler *et al.* (1997) as described in our previous study (Lugli *et al.* 2005), adding a cocktail of Rnase inhibitors to preserve RNA species. Synaptoneurosomal preparations were characterized by western blotting of PSD95 and by real-time RT-PCR measurements of BC1 and CAMK2a to verify that these components were enriched relative to total forebrain homogenate (see below). In the experiments reported here, each sample [synaptoneurosomes ('SYN') vs. total forebrain homogenate, 'total'] was pooled from 3–4 forebrains, and three independent pairs of samples were tested in parallel on microRNA microarrays. An additional three pairs of samples were prepared and measured for follow-up experiments.

About three-quarters (178 of 232) of the mouse microRNAs present on the microarrays were detectably expressed in the forebrain homogenate using the threshold criterion of having average intensity values ≥2.5 X background. Of the microRNAs expressed in forebrain, 78% were detectable within the SYN fraction using the same criterion. Normalizing the intensity values to the mean intensity values of the microRNA population as a whole (i.e., the mean value was chosen for one dye channel on one chip and the others normalized relative to that one) had only a slight effect on the distribution of intensity values (Fig. 2). However, this did improve the linearity of the assay and adjusted for possible variations among preps as well as for possible differences in the red versus green dye channels. Thus, this was the preferred method of normalizing microarray data.

Individual microRNAs showed a wide range of relative enrichment ratios (Fig. 3). The lowest ratio was observed with mir-143, which was ~ 7-fold less abundant in the SYN fraction than in the total homogenate, whereas on the other hand, mir-200c was ~ five-fold more abundant in the SYN fraction. Thirty-seven microRNAs showed an enrichment ratio of two-fold or greater in the SYN fraction, which in this experiment, was similar to the number of microRNAs (31) which achieved statistical significance at $p \leq 0.05$ (Table 1). Numerous microRNAs still achieved significance at more stringent criteria (Table 1).

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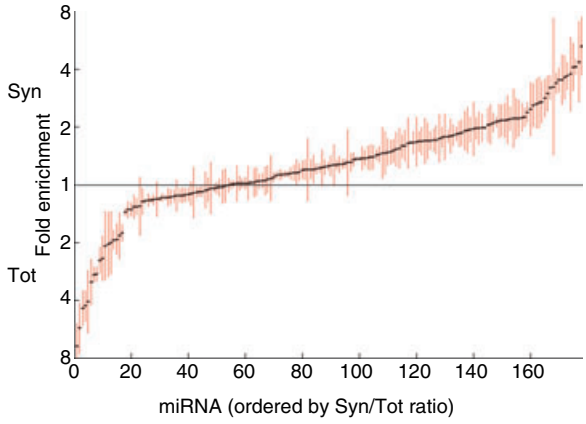

Fig. 3 Enrichment ratio ± SEM of forebrain microRNA intensity values (synaptoneurosomes/total homogenate) as measured by microarray.

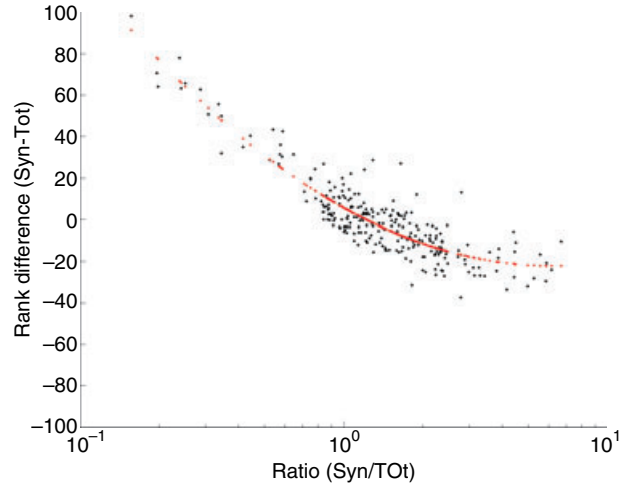

Fig. 4 microRNA enrichment in synaptoneurosomes versus total homogenate. Data were computed as the enrichment ratio (*X*-axis) versus computed as quantile rank differences (*Y*-axis). See text for details.

Table 1 Number of microRNAs (enriched in synaptoneurosomes) measured by microarray that achieve statistical significance at different stringency criteria

p-Value	# Observed microRNAs	# Expected by chance
0.10	65	12.4
0.05	31	6.2
0.01	7	1.24
0.005	4	0.62
0.001	2	0.12

Statistics were computed using the *t*-test (two-sided). Note that the Bonferroni correction for multiple testing is not appropriate for these data, since different microRNAs are not expressed independently of each other; see text.

Table 2 Enrichment ratios (synaptoneurosomes/total homogenate) of selected mature microRNAs measured by real-time qRT-PCR

microRNA	Mean ± SEM
miR-124a	1.04 ± 0.13
miR-125b	1.08 ± 0.22
miR-134	1.70 ± 0.59
miR-143	0.11* ± 0.022
miR-339	1.46* ± 0.13
miR-350	1.48* ± 0.07
miR-99a	1.79* ± 0.22

MicroRNAs were measured in 3–6 independent preps (three used in the microarray experiments and/or in three others prepared similarly). *Significantly enriched (or depleted) at a confidence level of $p < 0.01$ or better.

The synaptic enrichment was also analyzed by quantile ranking of the microRNAs. This is a non-parametric method in which each microRNA in a sample is simply ranked in order of its average intensity value, from greatest to least, and thus is assigned a quantile value (from 100% to 0%). Then, for each microRNA, one computes the difference between its rank value in synaptoneurosomes versus its rank value in the total forebrain homogenate. As shown in Fig. 4, there was a very good agreement between the findings obtained using enrichment ratios and obtained using rank differences.

Real-time RT-PCR measurements were made of selected microRNAs chosen because they covered a range of intensities and enrichment ratios (Table 2). MiR-124a, an abundant microRNA which is thought to be ubiquitously expressed in neurons (but not glial cells), had an enrichment ratio of 1.04, which is similar to the value estimated by microarray (0.88). Thus, miR-124a is neither enriched nor depleted from the SYN fraction, which is consistent with the lack of enrichment for other neuronal microRNAs measured by microarray (e.g., miR-128a, enrichment ratio = 0.85, Supplementary material File 2). The enrichment ratios measured by RT-PCR generally showed close agreement with the values determined by microarray for most microRNAs (Table 2), including that for miR-143 which was strongly depleted from the SYN fraction (0.11 vs. 0.15; Tables 2 and 3). A quantitative discrepancy between RT-PCR and microarray enrichment values was observed for miR-339, which showed less magnitude of enrichment ratio by RT-PCR (1.46, Table 2) than by microarray (4.07; Table 3). Similarly, miR-350 showed an enrichment ratio of 1.48 by RT-PCR in contrast to a measured value of 3.44 by microarray (Tables 2 and 3). This suggests that the microarray system did not maintain linearity throughout the measured range. Nevertheless, the qRT-PCR method did

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Table 3 microRNAs most and least enriched in synaptoneurosomes as measured by microarray

(syn/tot) Ratio	Syn intensity	Total intensity	miR	Expression
Top 20				
4.80	382	79	miR-200c	Vertebrate
4.07	819	200	miR-339	Rodent
4.03	315	78	miR-322	Rodent
3.72	408	109	miR-466	Vertebrate
3.69	255	69	miR-425	Vertebrate
3.55	852	239	miR-182	Vertebrate
3.44	2671	777	miR-350	Rodent
3.40	247	72	miR-183	Vertebrate
3.33	577	173	miR-351	Rodent
3.16	124	39	miR-297	Rodent
3.13	1546	494	miR-429	Vertebrate
2.98	253	84	miR-213	Vertebrate
2.83	702.	248	miR-325	Mammalian
2.68	1641	613	miR-200b	Vertebrate
2.65	1371	517	miR-337	Rodent
2.65	631	238	miR-323	Mammalian
2.64	15076	5706	miR-146	Vertebrate
2.49	944	378	miR-467	Rodent
2.34	5077	2168	miR-345	Mammalian
2.27	1903	840	miR-433-5p	Rodent
Bottom 20				
0.76	5489	7260	miR-218	Vertebrate
0.75	655	868	miR-219	Vertebrate
0.74	28233	38290	let-7b	*C. elegans* homolog
0.57	10211	17797	miR-125a	Vertebrate
0.55	2456	4460	miR-29c	Vertebrate
0.54	211	394	miR-148a	Vertebrate
0.53	1454	2749	miR-101a	Vertebrate
0.50	151	300	miR-377	Rodent
0.49	11006	22420	miR-29b	Vertebrate
0.48	96	201	miR-19b	Vertebrate
0.43	420	1003	miR-34c	*C. elegans* homolog
0.40	157	389	miR-34b	*C. elegans* homolog
0.35	9101	26059	miR-126-3p	Vertebrate
0.35	49	144	miR-126-5p	Vertebrate
0.32	4791	15041	miR-153	Vertebrate
0.25	557	2219	miR-301	Vertebrate
0.24	359	1473	miR-145	Vertebrate
0.23	3437	14784	miR-150	Vertebrate
0.18	281	1536	miR-451	Vertebrate
0.15	1960	13353	miR-143	Vertebrate

confirm that both miR-339 and miR-350 were significantly SYN-enriched (Table 2).

As shown in Fig. 5, SYN-enriched microRNAs exhibited a wide range of intensity values, and were NOT predominantly found among the least abundant microRNAs (this was checked since small changes in intensity values for microRNAs near threshold could potentially cause enrichment ratios to be artifactually inflated). This finding, together with

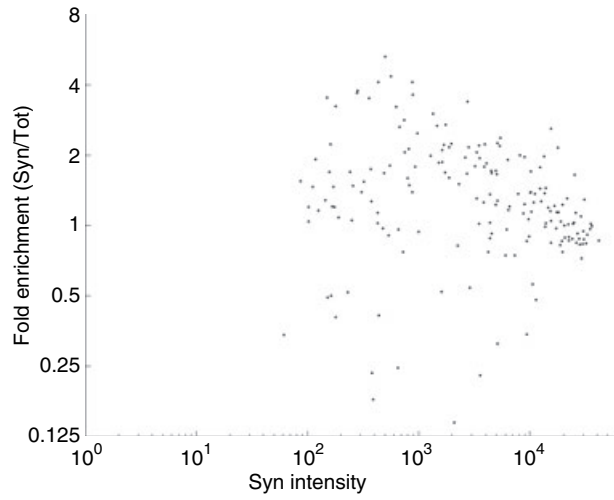

Fig. 5 Relationship between the microRNA intensity values observed in synaptoneurosomes and their enrichment ratios as measured by microarray.

the agreement between enrichment ratios and rank differences (Fig. 4), confirms that the observed enrichment of certain microRNAs in the SYN fraction is robust.

To analyze the biological features of SYN-enriched microRNAs, the top 20-most enriched microRNAs were compared to the bottom 20 (least-enriched) microRNAs (Table 3). The top 20 include eight microRNAs that were cloned originally from cultured cortical neurons (miR-322–352, Kim *et al.*, 2004), whereas none of these were found among the bottom 20. This is consistent with the expectation that synaptoneurosomes are enriched in pinched-off dendritic spines, which are derived from pyramidal neurons. Conversely, many of the bottom 20 microRNAs are expressed in many tissues and have been widely studied as being important in growth, differentiation and cancer (e.g., let-7b, miR-125a, 126, 143, 145, etc.). Several of the bottom 20 microRNAs have been reported to be expressed predominantly in astrocytes (miR-29b and 29c, Smirnova *et al.* 2005). The two sets of microRNAs differed in their evolutionary histories as well: The top 20 includes 11 microRNAs that have only been described in rodents and/or other mammalian species, and none have homologues in invertebrates. In contrast, the bottom 20 includes only 1 microRNA, their expression is restricted to mammals; most are expressed in fish and at least three have homologues in *C. elegans* (Table 3). Moreover, several of the bottom 20 microRNAs arise from multiple precursor genes (data not shown); it is likely that this reflects microRNA gene duplication and divergence over evolutionary time. In conclusion, the microRNAs that are enriched in synaptoneurosomes tend to be expressed predominantly in pyramidal neurons, and tend to be evolutionarily newer than forebrain microRNAs that are relatively depleted from the synaptic fraction.

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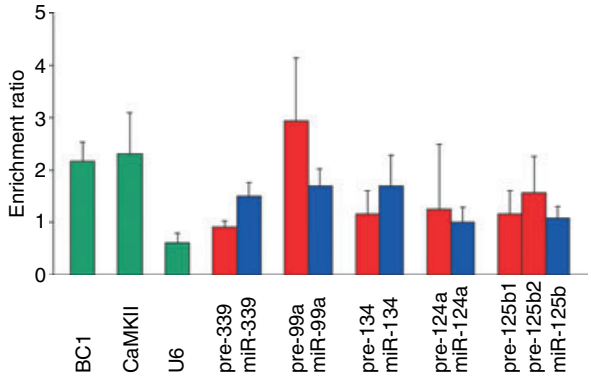

Fig. 6 Enrichment ratio (synaptoneurosomes/total homogenate) of various RNAs as measured by qRT-PCR. Data represent the average of three independent preps.

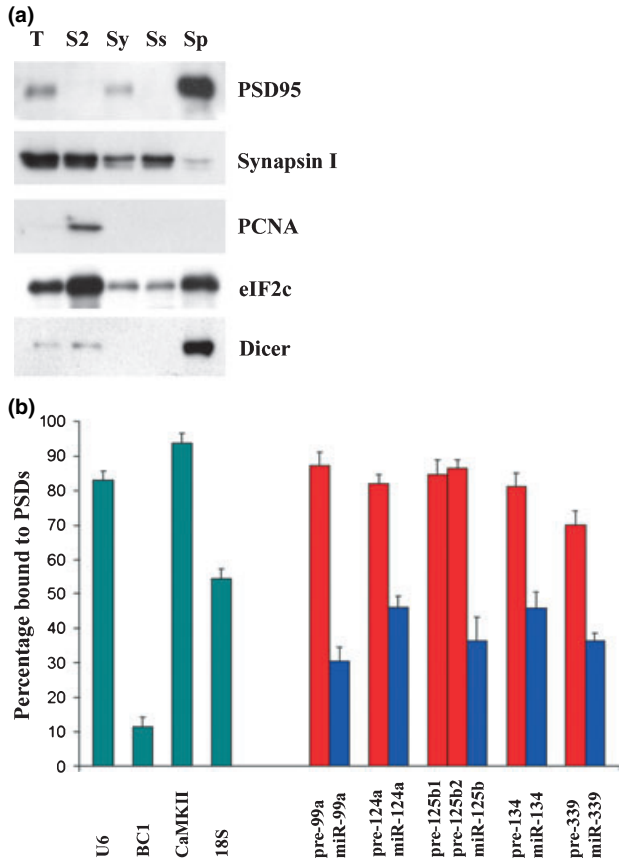

Fig. 7 Distribution of synaptosomal proteins and RNAs in soluble versus PSD fractions after extraction with Triton X-100. (a) Immunoblotting. Total forebrain homogenate (T) was processed to obtain a soluble cytoplasmic fraction (S2) and a synaptosomal fraction (Sy), that was then lysed with 1% TritonX-100 to yield (Ss) and insoluble fractions (Sp) as described in Materials and methods. Equal amounts of protein were loaded and blotted for different antibodies as indicated. The dicer antibody was chicken polyclonal anti-dicer (Lugli *et al.* 2005). (b) qRT-PCR measurements of RNAs. Total RNA was prepared from Ss and Sp fractions and measured as described in Materials and methods. The Sp/Sy ratio was calculated for three independent preps and is plotted as shown.

In many cases, two microRNAs encoded on the same chromosome at adjacent sites (presumably transcribed by the same primary transcript) showed very similar SYN enrichment ratios. For example, this was observed for three pairs of microRNAs within the top 20 (miR-322 and 351; miR-182 and 183; miR-429 and 200b) and four pairs within the bottom 20 (miR-29b and 29c; miR-34b and 34c; miR-126-5p and 126-3p; and miR-143 and 145). This underscores the consistency of the microarray results. Biologically, the phenomenon may simply reflect the fact that a primary transcript will be processed within the same cell type as the resulting mature microRNAs. Regardless, these cases are common and are important to note for data analysis, because they emphasize that the expression of any one microRNA cannot be assumed to be independent of the others.

Using real time qRT-PCR, the expression of selected microRNA precursors was compared in synaptoneurosomes versus the total forebrain homogenate. As shown in Fig. 6, microRNA precursors were readily detected within the SYN fraction, at levels that were comparable to the total homogenate. Because the primers derive from precursor sequences lying beyond the mature microRNA, this method should not detect mature microRNAs at all. On the other hand, the method detects both primary microRNA gene transcripts (pri-miRs) as well as small hairpin precursors (pre-miRs), as considered further in the section Distinguishing pre-miRs from pri-miRs in synaptic fractions.

Synaptosomes and post-synaptic densities

Synaptosomes are a well-studied synaptic fraction that is thought to consist predominantly of axon terminals with adherent PSDs. Detergent extraction of synaptosomes using Triton X-100 results in isolated PSDs. Synaptosomes and isolated synaptic densities were prepared and characterized as described in our previous publications (Smalheiser and Collins 2000; Lugli *et al.* 2005; see also Fig. 7a). Synaptosomes and isolated PSDs are more extensively rinsed than synaptoneurosomes and their isolation involves additional steps (e.g., sucrose gradients and detergent extraction) that should remove most contaminants. On the other hand, losses of RNA may occur because of the longer time involved in preparing synaptosomes and PSDs and/or to their extensive rinsing. We noted that levels of all RNAs measured, including ribosomal 18S RNA, U6, CAMK2a and BC1, as well as mature microRNAs and their precursors, were lower in synaptosomes relative to that observed in synaptoneurosomes. Because the components that were soluble (see below) were disproportionately lower in synaptosomes relative to synaptoneurosomes, it is likely that the losses predominantly reflect the more extensive rinsing of these preps (data not shown). Nevertheless, microRNA precursors

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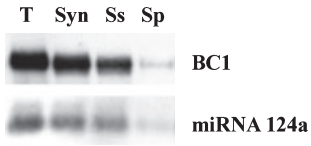

Fig. 8 Within synaptoneurosomes, BC1 RNA and mature miR-124a are predominantly soluble components. Total forebrain homogenate (T), synaptoneurosomes (Syn), and Triton-soluble (Ss) and insoluble (Sp) fractions of synaptoneurosomes were prepared. Equal amounts of total RNA were separated on agarose gels, transferred to Nylon membranes and blotted with biotinylated LNA oligonucleotide probes for BC1 and mir-124a, respectively (see Materials and methods). miR-124a was chosen as a representative microRNA because it is abundant, neuronal specific, and well characterized.

were readily detected within synaptosomes and PSDs and were amenable to study.

Synaptosomes were extracted with non-ionic detergent and measurements were made of the soluble extract versus the insoluble residue (i.e., the PSD fraction). As shown in Fig. 7b, the microRNA precursors were all predominantly associated with the PSD fraction. In contrast, the mature microRNAs were predominantly detected in the Triton-soluble fraction (Fig. 7b). The small RNA BC1 was almost entirely soluble as well. These results were confirmed for mature mir-124a and BC1 using Northern blotting of synaptoneurosomal lysates as well (Fig. 8).

To verify further that microRNA precursors are not simply contaminants of synaptic fractions that are co-isolated during subcellular fractionation, synaptosomes were lysed with RIPA detergent and immunopurification was carried out under stringent conditions using anti-PSD95 antibodies (Vinade *et al.* 2003). As a negative control, an antibody was employed against synapsin I, which is associated with pre-synaptic vesicles in this prep. Other negative controls included omitting primary antibody, and omitting synaptosomal lysate. The anti-PSD95 and anti-synapsin I antibodies did, as expected, bring down the respective proteins (Fig. 9a). As shown in Fig. 9b, anti-PSD95 brought down a striking amount of microRNA precursors (67.9-fold over baseline) and detectable, though small amounts of mature microRNAs (1.62-fold over baseline) both of which are significant at $p < 0.05$. Synapsin I antibody did not bring down significant amounts of either microRNAs or precursors (Fig. 9b).

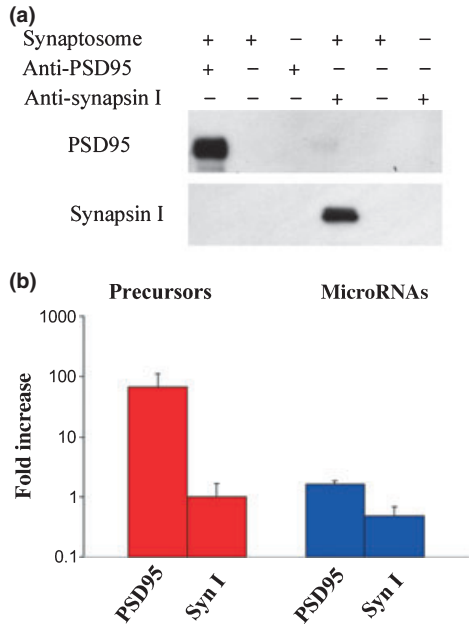

Fig. 9 Co-immunoprecipitation of microRNAs and their precursors with PSD95 or synapsin I. Synaptosomes were diluted 1 : 1 with RIPA buffer and incubated with anti-PSD95 or anti-Synapsin I (see Materials and methods). Negative controls include omitting antibody, or omitting synaptosomes. (a) Equal fractions were loaded and immnostained for PSD-95 and Synapsin I. (b) Total RNA was isolated from immunoprecipitates and measured for mature microRNAs (99a, 124a, 125b, 134, 143, 339) and their precursors (99a, 124a1-3, 125b1, 125b2, 134, 339) by qRT-PCR. Results were computed as the ratio between the amount of each RNA brought down by each antibody and the corresponding amount brought down in the baseline group lacking specific antibody. Because results were similar for all microRNAs and for all precursors, the data were pooled and displayed as red bars (precursors) versus blue bars (mature microRNAs).

Distinguishing pre-miRs from pri-miRs in synaptic fractions

The real time RT-PCR primers employed here do not distinguish between small microRNA hairpins (pre-miRs), which are ~ 70–110 nt. in length, and primary gene transcripts (pri-miRs), which are generally several kilobases long (Saini *et al.* 2007). Although pri-miRs are generally thought to be processed within the nucleus, there are some reports that pri-miRs can be transported intact to the cytoplasm under some conditions (Eis *et al.* 2005; Barthelson *et al.* 2007). As well, a noncoding RNA localized to the rat neuromuscular junction (Velleca *et al.* 1994) was identified as encoding a primary microRNA transcript (Rodriguez *et al.* 2004). Thus, pri-miRs might possibly be expressed in the cytoplasm or within synaptic compartments in the CNS as well.

It would have been desirable to pick primer pairs that are specific for selected pri-miRs from within the sequences flanking the pre-miRs, but the genomic structure of the microRNAs studied here have not been well characterized. As an alternative strategy, we employed the mirVana Paris kit (Ambion) to separate total synaptosomal RNA into pools of large and small RNAs, with a stated cut-off of ~200 nt. Large versus small RNA pools were measured by real time RT-PCR. Pre-miRs were chosen that have a simple relationship with the mature microRNA (i.e., multiple precursors do

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not exist for the same microRNA), that were readily measured by qRT-PCR, and that represented a wide range of SYN enrichment values, being on the top-20 list (miR-429, 146 and 100) or the bottom-20 list (miR-125a, 126, 153, and 301).

Using this method, miR-146 (a top-20 microRNA) and miR-125a (a bottom-20 microRNA) showed clean size separation, with the mature microRNAs being found predominantly in the small fraction and the pre-miRs predominantly in the large fraction. The pool of small RNAs contained 82% of the total amount of U6, 80% of the precursor activity for miR-146 and 88% of the precursor activity for miR-125a. This indicates that synaptosomes express precursors of miR-146 and 125a predominantly in the form of pre-miRs but little or no pri-miRs. In contrast, the pool of small RNAs within the total forebrain homogenate (which contains nuclear as well as cytoplasmic RNA) still contained most (86%) of the U6 RNA, but only 30% of the measured precursor activity for miR-146 and only 14% of the measured precursor activity for miR-125a. This suggests that, in the forebrain tissue as a whole, the pri-miRs for miR-146 and 125a are more abundant than are the pre-miRs.

The size selection method was evidently affected by RNA sequence and secondary structure; for example, we found that synthetic pre-miR-122a failed to partition into the small RNA pool both when tested by itself and when it was spiked into total forebrain RNA (data not shown). Similarly, we were not able to estimate the relative expression of pre-miRs versus pri-miRs in synaptosomes for some of the pre-miRs because they partitioned predominantly into the large RNA fraction, or showed partial sorting into both large and small fractions. Nevertheless, it was possible to estimate the relative synaptic enrichment of the entire set of selected pre-miRs by measuring their relative abundance in the small RNA pools of synaptoneurosomes versus total forebrain homogenate. The pre-miRs whose corresponding mature microRNAs showed high SYN enrichment (pre-mir-429, 146 and 100) exhibited significantly higher SYN enrichment as a group than did the pre-miRs whose corresponding mature microRNAs showed low SYN enrichment (pre-miR-125a, 126, 153, and 301; $p < 0.05$). This is consistent with the hypothesis that mature microRNAs expressed in synaptic fractions are likely to arise from local processing of their precursors.

Association of microRNAs and their precursors with protein complexes

Mature microRNAs are known to bind to the Argonaute homologue eIF2c as part of the RISC complex, and to be associated with FMRP as well (Caudy *et al.* 2002; Ishizuka *et al.* 2002). Several previous studies have also noted an association of FMRP with pre-miRs (Jin *et al.* 2004; Plante *et al.* 2006; Wulczyn *et al.* 2007), which is very interesting because FMRP or its homologues may potentially help to transport pre-miRs to dendrites (cf. Davidovic *et al.* 2007; who found that FMRP is a molecular adaptor between RNA granules and kinesin motors), and/or may potentially sequester pre-miRs from being cleaved by dicer. To investigate the association of microRNA precursors with proteins in brain *in vivo*, we prepared a low-speed S1 cytoplasmic fraction of forebrain homogenate and carried out immunoprecipitations using antibodies specific to a variety of microRNA pathway components, including dicer, eIF2c, FMRP, and PACT, as well as antibodies against PSD95, synapsin I, and MECP2. The immunoprecipitates were extracted for total RNA and measured for a series of selected mature microRNAs and microRNA precursors. (Note that the S1 fraction should exclude nuclear RNA where the majority of pri-miRs are thought to reside.) The immunoprecipitations were carried out under stringent conditions, i.e., in the presence of RIPA buffer. Some biologically significant interactions are disrupted by RIPA; for example, the previously reported association of dicer with eIF2c (Lugli *et al.* 2005) was not detectable in the presence of RIPA, whereas the association of dicer with FMRP was preserved (data not shown). Thus, binding that is detected in RIPA is likely to be specific and strong.

As shown in Fig. 10, FMRP showed a striking association with both microRNA precursors and mature microRNAs. Dicer showed a strong association with microRNA precursors as well, but no significant interaction with mature microRNAs.

Discussion

The present paper demonstrates that most of the microRNAs expressed within the adult mouse forebrain are readily detectable within synaptic fractions, at levels that are comparable to those seen within the total forebrain homogenate. In fact, 37 microRNAs were enriched two-fold or greater in synaptoneurosomes (SYN) as measured by microarray (Fig. 3), and the enrichment was confirmed for selected microRNAs by real time qRT-PCR (Table 2). The magnitude of SYN microRNA enrichment estimated by microarray (2–5-fold) was comparable to the enrichment observed with two known synaptic RNAs, BC1, and CAM kinase II alpha mRNA (Fig. 6). The forebrain microRNAs that were enriched in synaptoneurosomes were biologically quite distinct from microRNAs that were relatively depleted from this fraction, both in their tissue expression patterns (many were expressed predominantly in pyramidal neurons) and in their evolutionary histories (SYN-enriched microRNAs tended to be evolutionarily new, often mammalian-specific or rodent-specific). These findings strongly suggest that a broad, diverse, yet biologically coherent population of microRNAs is expressed within dendrites and within dendritic spines, where they may be expected to contribute to the regulation of local protein synthesis.

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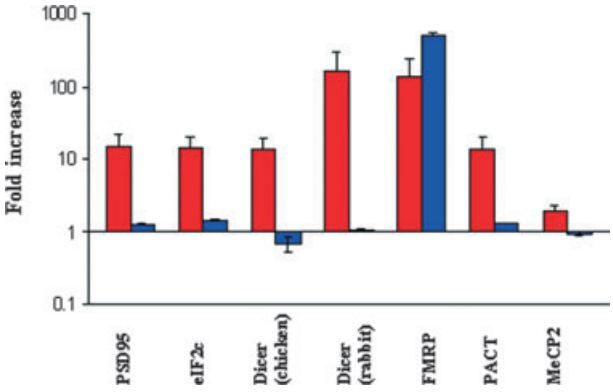

Fig. 10 Co-immunoprecipitation of mature microRNAs and microRNA precursors with different proteins. Total forebrain homogenate was spun at low speed to remove nuclei and then the S1 low speed cytoplasmic supernatant was prepared (1500 *g* for 10 min; final concentration 1.8 mg/mL). This was diluted 1 : 1 with RIPA buffer; each tube had 800 μL final volume. Immunoprecipitations were performed with a variety of affinity purified antibodies (commercially available or made and characterized in Lugli *et al.* 2005): (a) mouse monoclonal anti-PSD95 (clone 28/86); (b) rabbit polyclonal anti-eIF2c that recognizes multiple isoforms; (c) chicken polyclonal anti-dicer recognizing a region (1389–1404) located between the first and second RNAse III domains; (d) rabbit polyclonal anti-dicer recognizing the C-terminus; (e) mouse monoclonal anti-FMRP (Brown *et al.* 2001; clone 7G1-1, Developmental Studies Hybridoma Bank, Iowa City, IA); (f) rabbit polyclonal anti-PACT (ProteinTech Group Inc., Chicago, IL, USA); (g) rabbit polyclonal anti-MeCP2 (Upstate Biotech, Lake Placid, NY, USA). As a negative control, rabbit polyclonal anti-Synapsin I was used (Chemicon, AB1543P). All antibodies brought down their respective proteins as expected (Figs 7 and 9 and data not shown). However, no detectable GAPDH or CAMK2a mRNA were detected in any groups (data not shown). Total RNA was extracted and each immunoprecipitate was measured for mature microRNAs (99a, 124a, 125b, 134, 143, 339) and their precursors (99a, 124a1-3, 125b1, 125b2, 134, 339) by qRT-PCR. Results were computed as the ratio between the amount brought down by each antibody and the corresponding amount brought down by anti-synapsin I. Because results were similar for all microRNAs and for all precursors, the data were pooled and displayed as red bars (precursors) versus blue bars (mature microRNAs). Note the log scale.

MicroRNA precursors were also readily detected in synaptoneurosomes at levels similar to or greater than that found in the total forebrain homogenate (Fig. 6). Within synaptosomes, the microRNA precursors were predominantly associated with PSDs (Fig. 7). This was further confirmed by co-immunoprecipitation with PSD95 protein (Fig. 9). Although the real time RT-PCR method used here does not distinguish between primary microRNA gene transcripts (pri-miRs) and small hairpin precursors (pre-miRs), size fractionation experiments indicate that, at least in the case of miR-146 and 125a, pre-miRs are the predominant form within synaptic fractions. The available data do not exclude that pri-miRs may be expressed near synapses in some cases. However, we conclude that microRNA precursor forms (and not merely mature microRNAs) are prominent synaptic components.

The findings reported here support our earlier proposal that pathways related to RNA interference regulate long-term gene expression in the mature brain (Smalheiser *et al.* 2001). They also extend the observations that the RNAse III enzyme dicer is expressed within dendritic spines and is enriched in PSDs, in a form that is enzymatically inactive (Lugli *et al.* 2005; see also Fig. 7a). It is unlikely that dicer bound to PSDs lacks a necessary co-factor needed for processing, since the RNAse III enzymatic assay (Lugli *et al.* 2005) employed exogenous dsRNA which does not require any co-factors. As well, at least one known dicer co-factor, PACT, is detectably associated with isolated PSDs (unpublished data). NMDA stimulation of hippocampal slices or raising intracellular calcium levels within synaptoneurosomes causes the calpain-dependent liberation of enzymatically active dicer (Lugli *et al.* 2005). Thus, dicer and pre-miRs are both located at PSDs but are prevented from processing further under resting conditions, apparently because of the presence of endogenous inhibitor(s) of dicer activity and/or pre-miR processing (cf. Leuschner and Martinez 2007) that are removed under stimulated conditions (e.g., by calpain, by another calcium-dependent protease or by a phosphorylation event).

The present findings also support the suggestion that the FMRP may be involved in the processing arm of the microRNA pathway (associated with transport and/or processing of pre-miRs) as well as the effector arm (associated with mature microRNAs, RISC, and target mRNAs). MicroRNA precursors were strongly associated with both dicer and FMRP in cytoplasmic extracts, and dicer interacted with FMRP, as shown by co-immunoprecipitation with specific antibodies under stringent conditions (Fig. 10). Thus, it is likely that pre-miRs, dicer and FMRP are part of a single complex *in vivo*. Studies of whole brain in neonatal *fmr1* knockout mouse have shown no deficits in overall microRNA levels (Landgraf *et al.* 2007), and our preliminary studies have confirmed that no global changes in microRNA levels are seen either in adult mouse forebrain or within synaptoneurosomes (G. Lugli *et al.*, in preparation). However, this does not rule out a role for FMRP in pre-miR processing, because FMRP homologues FXR1P and/or FXR2P may also interact with mature microRNAs and microRNA precursors (Jin *et al.* 2004; Plante *et al.* 2006; Duan and Jin 2006). As well, we have observed alterations in specific microRNAs in *fmr1* knockout mouse that vary according to brain region and that can affect synaptic fractions differently than whole tissues (Lugli *et al.*, in preparation). Thus, further studies are needed to learn whether FMRP homologues may play a role in pre-miR processing in conjunction with dicer at PSDs, and especially under conditions of localized synaptic stimulation that elicit long-term plasticity.

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Finally, how might the microRNA machinery described here contribute to the regulation of protein translation near and within dendritic spines, which has been intensively studied by neurobiologists as a fundamental mechanism of synaptic plasticity? Certainly, localized protein synthesis occurs within dendritic spines: mRNAs and translational regulatory components have been detected in close proximity to PSDs (Asaki *et al.* 2003; Suzuki *et al.* 2007), and intense stimuli that elicit long-term potentiation (LTP) have been shown to cause some polyribosomes to move into dendritic spines where they are situated very close to active synapses (Ostroff *et al.* 2002; Bourne *et al.* 2007). Such conditions are also associated with localized increases of calcium and activation of calpain. We propose that synaptic stimulation may lead to local processing of pre-miRs by dicer near the post-synaptic density, leading to the loading of RISC with mature microRNAs that bind to any available target mRNAs that are restricted to the activated synapse.

However, it is not clear whether microRNAs should be expected to repress translation or to enhance it: Whereas microRNAs do appear to repress translation of mRNAs within neurons under resting conditions (Kosik 2006; Schratt *et al.* 2006), it has been recently discovered that RISC can actually ENHANCE translation of a target mRNA as a function of the cell cycle, depending on the facultative binding of a FXR1 isoform to RISC (Vasudevan *et al.* 2007). It is not too hard to conceive that a similar switch might occur reversibly in the context of intense synaptic stimulation. Indeed, although FMRP (like microRNAs) is thought of as a translational repressor, FMRP is required for a transient burst of translation of specific synaptic target mRNAs to occur upon stimulation by mGluR5 agonist (e.g., Weiler *et al.* 1997; Muddashetty *et al.* 2007). This apparent paradox may be explained if FMRP works together with microRNAs in a concerted but transient switch from repression to enhancement of target mRNA translation. Upon mGluR5 stimulation, FMRP undergoes a rapid but transient dephosphorylation that might be related to this transient burst of translation (Narayanan *et al.* 2007). Further research is needed to learn whether processing of pre-miRs near synapses, expected to accompany LTP-eliciting stimuli, will have transient or long-lasting effects on their target mRNAs, and whether the effects will be repressive or stimulatory.

It is even less certain what the fate may be of synaptic microRNAs that become loaded onto RISC but that do *not* encounter target mRNAs. Are these rapidly destroyed? Or do they persist for hours (or longer) within dendritic spines? If they have a significant lifetime, then potentially they may bind mRNAs that are being transported down dendrites and 'trap' them to the vicinity of previously activated synapses. This could potentially serve as a type of synaptic tagging and capture (cf. Martin and Kosik 2002; Barco *et al.* 2008).

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Supplementary material

The following supplementary material is available for this article:

File S1. List of primer sequences used for RT and PCR reactions.

File S2. Entire list of measured human, rat and mouse microRNAs by microarray after filtering and normalization.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1471-4159.2008.05413.x (This link will take you to the article abstract).

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