miRNA profiling of bilateral rat hippocampal CA3 by deep sequencing

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Abstract

MicroRNAs (miRNAs) have been demonstrated to be potent post-trascriptional modulators of protein expression. miRNA expression was profiled in the left and right dorsal hippocampal CA3 of mature rats by high-throughput deep sequencing. Among the sequenced and cross-mapped small RNAs, 88% belonged to the miRNAs annotated in the miRBase 15 database. Nearly half of the small RNAs belonged to the let-7 family miRNA. Seven percent of the sequenced small RNAs were not annotated in miRBase 15. Bioinformatic analysis of the unannotated small RNA sequences suggested seventeen novel miRNA candidates with relatively high expression levels (> 100 tags per million). The left:right expression ratios were similar for all highly expressed miRNAs with less than 10% differences. These results provide a basic idea of the relative expression strengths of known and unknown miRNAs in the dorsal hippocampal CA3.

Keywords

deep sequencing, hippocampus, left-right asymmetry, let-7

Running Title

MicroRNAs of bilateral hippocampal CA3 in rats
1. Introduction

MicroRNAs (miRNAs) are evolutionally preserved short (18 to 25 nucleotide) RNAs that bind to the 3’ untranslated regions (UTR) of mRNAs to interfere translation (for a review [1]). Since the original discovery in nematodes [2], several hundred miRNAs have been identified in rodents. Some miRNA are enriched in particular cell types in the nervous system. For example, in the murine stem cell-derived system, miR-124 and miR-128 are specifically expressed in neurons [3], whereas miR-23, miR-26 and miR-29 are preferentially expressed in glia during cell differentiation [4]. Interestingly, miRNA expression are shown critical in coordinating the left-right axis of the nervous system in nematodes [5].

In rodents, the left and right hippocampi are interconnected by massive glutamatergic projections from CA3 pyramidal cells and hilar cells. Functional and morphological characteristics of hippocampal CA3-CA1 synapses have been shown to be biased by the laterality of CA3 neurons [6, 7]. Such asymmetrical distribution of CA3-CA1 synapses has been suggested to be in part genetically programmed [8]. Screening of genes by cDNA microarrays suggested some genes are more abundantly expressed in one side of the hippocampus [9-11], yet genes that pronouncedly sculpt the left-right asymmetry of mammalian hippocampus have not been identified.

Recent advances of high-throughput sequencing allow quantitative assessment of transcribed RNAs. Expression levels of miRNAs can be measured by sequencing PCR-amplified, linker-tagged small RNA libraries [12, 13]. In this study, we investigated the miRNA expression profiles in both sides of the CA3 regions by high-throughput (deep) sequencing.
2. Material and Methods

2.1 Preparation of CA3 samples

RNA was purified from five week old male Long-Evans rats. For each rat, the brain was quickly removed from the skull after decapitation and dissected in half at the midline. The brain was then immediately cooled in ice-chilled Ringer solution. The left and right brains were cooled in different containers. After cooling for at least 3 minutes, the brain was taken out of the cooling solution and the hippocampus was gently isolated using spatulas. Coronal sections (400 µm thickness) were prepared using a tissue chopper. Region CA3 was dissected under a stereomicroscope using custom-made RNase free razor blades. Isolated CA3 tissue was briefly triturated in TRIzol (15596-026, Invitrogen) and frozen in liquid nitrogen. The samples were collected in alternative sequences of laterality (i.e. left, right; right, left; left right ...) so that the sampling time bias contributing to laterality was minimized. The collected samples were stored frozen in -80 °C.

All experimental procedures involving animals were approved by the RIKEN Animal Experiments Committee.

2.2 Purification of small RNA and construction of small RNA library

On the day of RNA purification, CA3 samples from individual animals were defrosted on ice and mixed to compose three sample sets. The total number of animals used in each sample was 36, 57, and 34. RNA was extracted according to the manufacturer's instructions (Invitrogen) by DNAform KK (Yokohama, Japan). On average, we could obtain a total RNA amount of 2.2 µg per animal per dorsal hemisphere hippocampal CA3. As the extracted RNA elution generally had high 230/260 values of >1.4, additional purification by the miRNeasy kit (Qiagen) was performed before
constructing small RNA libraries. We used a total RNA amount of 3.0 µg per sample for this purification. Small RNA libraries were made as described previously [14]. The concentration of each purified small RNA cDNA library was determined using an Agilent 2100 BioAnalyzer with a DNA1000 Labchip (Agilent) and adjusted to 6 pM for sequencing. The small RNA libraries were sequenced using a Genome Analyzer IIx (Illumina).

2.3 Data Analysis

Sequence extraction and mapping was done as described in [15]. All sequencer reads containing undetermined bases (N) were considered to be low quality and were discarded from further analysis. Of the remaining reads, only the ones containing the illumina-specific 3' linker, ATCTCGTATGCCGTCTTCTGCTTG, and the barcode GAAA perfectly were selected. We subtracted linker sequences and the barcode from the reads and assigned each tag to an individual sample based on the barcode. Extracted tags between 15 and 50 bases were used for further analysis.

All small RNA tags were mapped to the genome assembly rm4, except for haplotype and random sequences, and ribosomal sequences accession number V01270.1 using Eland, the default Illumina mapping tool with standard parameters (up to 2 mismatches allowed). Tags mapping with fewer mismatches to ribosomal sequences than the genome were discarded. Artifact sequences from tags are removed by the Tagdust program (v. 1.0) using parameter false recovery rate=0.01 against library sequences:

\[
\begin{align*}
\text{GTTCAGAGTTCTACAGTCCGACGATGAAAATCTCGTATGCCGTCTTCTGCTTG,} \\
\text{GTTCAGAGTTCTACAGTCCGACGATATCTCGTATGCCGTCTTCTGCTTG,} \\
\text{CAAGCAGAAGACGGCATACGA,} \\
\end{align*}
\]
AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA (barcode sequence is in brackets). We applied a previously described correction algorithm [15] to avoid cross-mapping between highly similar small RNAs. We applied the miRDeep algorithm [16] to small RNA candidates whose mapping location do not have any genome annotation. For each cluster of unannotated tags on the genome, the possible pre-miRNAs were selected, and RNAfold was used to discover any hairpin sequences. Subsequently, the miRDeep core algorithm was applied to detect which of the hairpins contain a potential novel miRNA. The output of miRDeep gives the genome locations, the mature miRNA, the pre-miRNA, and other relevant information.

For computation of the left:right ratios of miRNA expressions, each miRNA expression was first normalized to the total clone counts of the previously annotated miRNAs. Logarithmic values of the left:right ratios of miRNA expressions were computed for each sample sets and the mean values were computed.

In the main text, numbers are presented as mean ± standard deviation unless otherwise noted.

2.4 In situ hybridization of miRNA

In situ hybridization (ISH) was performed using digoxygenin (DIG)-labeled LNA probes according to the protocol described in Pena et al. [17] with minor changes. LNA probes used for ISH are as follows: miR124: [C]ce[T]ctgg[T]ca[A]ccag[T]ca[C]a, miRNA candidate 1: gg[T][T]ag[A]ac[T]tg[A]t[AG]c[T]cca[A], miRNA candidate 2: [G]tt[G]c[A]g[AG]t[C][T][C]aaa[T]c[A]. LNA bases are shown in square-bracketed capital letters. In some experiments, immunohistochemistry was performed after hybridization. Primary antibodies were applied for 1 hour before incubation in
anti-DIG-FAB-POD. In double staining experiments, anti-DIG-FAB-POD antibody and Alexa488-conjugated secondary antibody (1:1000, Invitrogen) were applied together.

3. Results

Three sets of left and right hippocampal CA3 RNA cDNA libraries were analyzed by deep sequencing. Between 9.8 and 10.8 million tags were read from each samples totaling to more than 60 million sequenced small RNAs. Of these sequenced small RNAs, 93.3 ± 1.1 % was mapped to previously identified miRNAs (miRBase release 15, www.miRBase.org) [18] or other small RNA sequences using a cross-mapping procedure [15]. The coefficient of variation (C.V.) of normalized clone count for each miRNA was computed (n = 6, left and right samples combined). When plotted against the mean normalized clone count (Fig. 1A), the C.V. appeared to be invariant with the clone count for miRNAs with larger than 100 tags per million (tpm; mean C.V. = 0.23 ± 0.11, Pearson correlation coefficient r_P = -0.067), indicating reliable quantification of miRNAs for a wide range of expression.

Overall, 72 miRNAs out of 427 mapped small RNAs had clone counts of more than 1000 tpm. The top twenty miRNAs in clone counts occupied approximately 72.9% of total small RNA clones (Table 1, the complete expression profile is in Supplemental Table S1). Notably, the let-7 family occupied 49.0% of the clone counts (Fig. 1B), whereas the star sequences for let-7 family totaled to less than 0.02%. Among the let-7 family, let-7c was the major miRNA representing 47.9%. let-7a, let-7b, let-7f represented 18.6%, 13.3%, 18.0%, respectively. let-7d, let-7e, and let-7i were mapped with less than 1% each. Other let-7 family miRNAs were not identified in our samples.
Non-miRNA small RNAs (mostly tRNA and rRNA) were less than 2%. To make sure that the dominance of the let-7 family miRNAs is not an artifact of the cross-mapping procedure, we extracted the miRNA counts that exactly mapped the mature rat miRNA annotated in miRBase using Perl with BioPerl extension. On average there were 4.9 ± 0.41 million of such clones in each sample, accounting for about a half of the clone counts. let-7c, let-7a, let-7b and let-7f occupied 35.2%, 13.6%, 7.1%, and 13.4%, totaling to 69.3% of the exactly mapped miRNAs. In this analysis, amongst other relatively highly expressed miRNAs (i.e. > 1.0%) were miR-9 (5.6%), miR-126 (2.4%), miR-29b (1.5%), miR-24 (1.4%), miR-103 (1.3%), miR-27b (1.3%), miR-26a (1.0%). These miRNAs had a comparable presence in the cross-mapped expression profile.

We next compared the expression profile of the rat CA3 with that of the whole mouse hippocampus published by Pena et al. [17]. The Spearman correlation coefficient (\(r_S\)) between the rat and mouse data was calculated to be 0.70 (\(p < 10^{-9}\)) when the rat miRNAs with larger than 0.1% clone counts with matching murine miRNA were considered (Fig. 2). A similar correlation value was computed for the 200 most abundantly expressed rat miRNAs (\(r_S = 0.71, p < 10^{-24}\)). These results show that the relative miRNA expression profiles are similar for rats and mice.

Approximately 7% of the small RNA clones were mapped to genomic locations previously unannotated in miRBase 15, mRNA, rRNA, tRNA, or non-coding RNA (‘Unknown’ in Fig. 1B). We further analyzed these clones by the miRDeep software [16]. Thresholding the total miRDeep score with the value 1 resulted in 337 novel miRNA precursor sequence candidates, among which 41 had expression levels above 100 tpm. Excluding mature sequences that had similar annotated sequences in other species, there were seventeen novel mature miRNA candidates (Table S2). Two
miRNA candidates, UUGGAGUUCAUGCAAGUUCUAACC (*miRNA candidate 1*) and UGAUUGGAAGACACUCUGCAAC (*miRNA candidate 2*), had clone counts larger than 10,000 tpm. ISH was performed to compare the localization of the novel miRNAs with a previously known miR124 [17] expression pattern (Fig. 3). ISH of miR-124 showed a Nissl-staining-like pattern in which bright labeling delineated principal cell layers of the hippocampus along with scattered labeling in other layers (Fig. 3A). Immunohistochemistry against an astrocyte marker protein S100B, shows that miR-124 signal is minimal in astrocytes. Cells that are brightly labeled in stratum radiatum (Fig. 3B) are presumed to be interneurons, as they have larger somata than astrocytes. IHC of the two novel miRNA candidates showed strikingly different labeling patterns (Fig. 3 B and C). Unlike miR-124, ISH for *miRNA candidate 1* hardly labeled bright cell bodies outside the pyramidal cell layer, whereas ISH for *miRNA candidate 2* labeled cell bodies outside the pyramidal layer to a similar degree as miR-124. Interestingly, ISH for *miRNA candidate 1* labeled neuropil outside the pyramidal cell layer, suggesting the possibility of transport of the molecule to dendrites. The inner part of the granule cell layer is more brightly labeled for both miRNA candidates, hinting a developmental modulation of the miRNA. For both miRNA candidates, subpopulations of principal cells were brightly labeled, whereas ISH for miRNA-124 labeled virtually all principal neurons.

Finally, we compared the miRNA expressions in the left and right CA3 by computing the Pearson correlation coefficients ($r_p$) of log-transformed miRNA expression strengths. We found that the left and right miRNA expressions are extremely well-correlated for each sample set ($r_p = 0.99, 0.99, 0.99$). Plotting left vs. right miRNA expression resulted in data points in the neighborhood of the 45 degree line (Fig. 4A). The high correlation and similar expression value were observed
throughout the expression range (Fig. 4B, insets). There are only a few data points that deviate from the 45 degree line, however, these points are either of low clone counts or the error bars are large. To quantify the balance of the left and right miRNA expression, we computed the logarithmic value of the left:right expression ratio \( \ln(L/R) \) for each miRNA that had clone counts larger than 100 tpm in at least one sample. We found that the distribution of \( \ln(L/R) \) is bell-shaped with a mean of 0.022 (left:right ratio of 1.02) and a standard deviation of 0.078 (n = 164) (Figure 4B). Among the dataset, miRNAs that had left:right or right:left ratio larger than 1.2 were miR-9*, miR-125b-3p, and miR-30e. A close look at the original clone counts showed that miR-125b-3p and miR-30e had generally low clone counts (< 100 tpm) and only one sample set had a strong bias. None of the novel miRNA candidates had both clone counts of greater than 100 tpm (131) and consistent left:right (or right:left) asymmetry of greater than 10%.

4. Discussion

Surprisingly high proportions of the let-7 family miRNAs were found in our samples compared with previously published deep sequencing results in the mouse whole brain [19] or hippocampus [17]. The discrepancy between our results and others is not clear. One potential cause of the difference is the use of LNA oligonucleotide in our experiments to remove adapter dimers and lesser number of PCR cycles (12 vs. 20 [20, 21]) to construct the small RNA library. It is noted that more recent deep sequencing data of the mouse hippocampus from another group also shows high amounts of let-7 family [22].
Originally discovered as the \textit{lethal-7} gene that suspends stem-cell-like divisions of the seam cells in \textit{C. Elegans} \cite{23}, the let-7 family of miRNA is highly conserved across animal species including rodents and humans. The let-7 family miRNAs play a critical role during cell differentiation involving \textit{lin-28} and \textit{lin-41} \cite{24, 25} and have been shown to be upregulated towards later stages of mouse brain development \cite{26}. It is conceivable that the abundance of let-7 family miRNAs merely reflects that hippocampal CA3 cells are already differentiated and mature. Interestingly, let-7 family miRNAs were recently shown to repressively regulate the \textmu-opioid receptors \cite{27}. It is possible that a considerable proportion of \textmu-opioid receptor mRNA is interfered at the 3’-UTR.

Many of the miRNAs that were reported to be influential in neural function were expressed in relatively high amounts in CA3. For instance, miR-9 had a relative expression level of approximately 50,000 tpm. miR-124, miR-125, miR-128, miR-129 all had expression levels greater than 1,000 tpm. Interestingly, miR-134, which has been reported to play a role in synaptic plasticity and dendrite formation of hippocampal neurons \cite{28-30}, did not appear to have a prominent presence. A recent study estimated that a mouse liver cell contains \textasciitilde120,000 miRNAs and a human hematopoietic progenitor cell contains 12,000 miRNAs \cite{31}. If we assumed 100,000 miRNAs in a neuron, there would be on average fifty miR-134s. Considering that miRNAs generally target UTRs of multiple mRNAs, whether such a number is realistic to mediate synaptic plasticity is a subject of future studies.

Bioinformatic analysis of unannotated clones resulted in seventeen novel miRNA candidates. Interestingly, the two miRNA candidates that had greater than 10,000 tpm had characteristically different localization patterns, hinting sub-cellular localizations
and cell-type specific expression of the miRNA candidates. Notably, the *miRNA candidate 1* sequence is now found as murine miR-3068 in the latest release of miRBase (release 16) that came out shortly after our analyses. The structural and biological significance of these miRNA candidates are not addressed in the current study, but the differential ISH patterns are encouraging for further investigations.

Comparison of the left vs. right miRNA expression did not result in identification of any miRNAs that characterize the laterality of the hippocampus, supporting a previous microarray-based miRNA profiling study of larger regions of the rat brain [32]. Our data suggest that miRNAs have little to do with maintaining the lateral identity in the asymmetric hippocampal circuitry. It remains possible that the left-right asymmetry is defined during early brain development and miRNA expression in the mature brain does not reflect the asymmetry. Further miRNA profiling throughout the brain developmental stages is needed to address such issue.

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The sequencing results are archived in the DDJB Sequence Read Archive (DRB) database (accession number: DRA000379) [http://trace.ddbj.nig.ac.jp/DRASearch/submission?acc=DRA000379]

References


Figure 1. Reliability and distribution of sequenced small RNAs from rat hippocampus region CA3.

A. Coefficient of variation (C.V.) of normalized clone count is computed from six samples and plotted against the mean clone counts for each miRNA with > 10 tpm.

B. Distribution of sequenced small RNAs from rat hippocampal CA3. Note that half of the mapped miRNAs belong to the miRNA-let-7 family. Inset: Distribution of the let-7 family miRNAs in rat hippocampus region CA3.
Figure 2. miRNA expression profile comparison of the rat hippocampal CA3 vs. mouse whole hippocampus. Proportions of miRNAs with tpm > 1,000 are plotted against the published data for whole mouse hippocampus [17].
Figure 3. Novel miRNA candidates show different in situ hybridization patterns in the hippocampus. A. In situ hybridization pattern of miR-124 (in orange) shows a Nissl-stain-like pattern. Virtually all principal cells are labeled. Counter-immunostaining with the astrocytic marker S100B (in green) reveals that the localization of miR-124 is predominantly in neurons, as seen in the magnified view of the cyan rectangle (CA3 stratum radiatum) in A’. Arrows and arrowheads indicate presumed interneurons and astrocytes, respectively. In situ hybridizations of two novel miRNA candidates are shown in B and C. B’ and C’ represent magnified views of the CA3b area. Scale bars: A: 1 mm, A’: 40 µm, B&C: 500 µm, B’&C’: 100 µm.
Figure 4. Comparison of miRNA expression in left vs. right hippocampal CA3.  A. Scattergram for left vs. right expression is plotted for each previously annotated miRNA. The main chart covers the expression range between 100 and 10,000 tpm. Left and right insets covers the expression ranges [1,100] and [10⁴, 10⁶] tpm, respectively. B. Histogram for lateral proportion of miRNA expression. Only miRNAs with more than 100 tpm in either side of hippocampal CA3 are evaluated. The left half of the graph represents the frequencies of right:left ratios and the right half represents that of left:right ratios. Error bars are standard errors of the means in both directions.
Table 1. List of miRNAs with high clone counts (let family and twenty other miRNAs). See Suppl. Table S1 for full listing.

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<th>miRNA ID</th>
<th>Relative frequency (tags/million)</th>
<th>Cumulative sum (%)</th>
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<tr>
<td>let-7c</td>
<td>234979.7</td>
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<td>mir-138</td>
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<td>25228.3</td>
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