Neuronal Activity Regulates Hippocampal miRNA Expression

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Abstract

Neuronal activity regulates a broad range of processes in the hippocampus, including the precise regulation of translation. Disruptions in proper translational control in the nervous system are associated with a variety of disorders that fall in the autistic spectrum. MicroRNA (miRNA) represent a relatively recently discovered player in the regulation of translation in the nervous system. We have conducted an in depth analysis of how neuronal activity regulates miRNA expression in the hippocampus. Using deep sequencing we exhaustively identify all miRNAs, including 15 novel miRNAs, expressed in hippocampus of the adult mouse. We identified 119 miRNAs documented in miRBase but less than half of these miRNA were expressed at a level greater than 0.1% of total miRNA. Expression profiling following induction of neuronal activity by electroconvulsive shock demonstrates that most miRNA show a biphasic pattern of expression:rapid induction of specific mature miRNA expression followed by a decline in expression. These results have important implications into how miRNAs influence activity-dependent translational control.

Introduction

Translational control is essential for the normal function of neurons. Numerous mechanisms exist in neurons to finely tune the translational output of mRNAs [1]. These mechanisms are required for the establishment long-term memories and defects in translational control lie at the heart of a variety of syndromes that display neuropathological and neurocognitive defects. Among these is Fragile X Syndrome, caused by the mutation of the Fragile X Mental Retardation Protein (FMRP), an RNA binding protein involved in both the transport and translation of specific sets of mRNAs [2]. Tuberculosis is caused by mutations in the Tuberous sclerosis complex 1 or 2 genes, which in turn regulates mechanistic target of rapamycin- (mTOR) dependent translation. These syndromes directly affect translational control in neurons and lead to cognitive disorders that lie within the autistic spectrum [3].

MicroRNA (miRNA) play a significant role in the translational control of mRNAs in neurons. These small RNAs are loaded into the RNA-induced silencing complex (RISC) where they direct target mRNAs in a sequence-specific fashion for either translational repression or degradation [4]. Both the mTOR and FMRP pathways regulate RISC’s repressive activity. Activation of the mTOR pathway by neurotrophin signaling results in the derepression of miRNA-silenced Lim kinase 1 mRNA [5]. The Drosophila homolog of FMRP (dFmr1) physically interacts with components of RISC [6] and dPum and the core RISC component dAgo1 show genetic interactions [7]. These interactions may also be relevant in mammals as FMRP immunoprecipitates a subset of neuronally enriched miRNAs including miR-125b and miR-132 [8]. These observations together suggest that miRNA-mediated translational repression could play a significant role in Tuberous sclerosis and Fragile X-associated neurocognitive defects. Disruptions in the biogenesis of miRNAs also contribute to the behavioral deficits observed in the mouse model of 22q11.2 microdeletion syndrome [9]. In all, it is likely that the miRNA system plays a significant role in the homeostasis of neuronal protein synthesis, that when disrupted leads to cognitive disorders.

Neuronal activity regulates protein synthesis in a variety of ways including the mobilization of translationally repressed mRNAs, including those regulated by mTOR and FMRP. Though a number of activity-regulated miRNAs in the hippocampus have been identified [10,11,12], a comprehensive approach to profiling of miRNA expression in response to neuronal activity in the
hippocampus has yet to be described. The majority of these studies have focused on miRNAs that are induced by activity, a phenomenon seemingly at odds with enhanced protein synthesis observed in neurons following activation. In this study we use multiple expression profiling platforms to comprehensively identify all miRNA expressed in the hippocampus, the essential structure of the brain required for learning and memory. Additionally, we use high- and low-throughput methods to describe how neuronal activity impacts the expression of miRNAs. Our observations demonstrate that some miRNAs are induced by activity while nearly all miRNAs show a long period of decline in expression following neuronal activation. These results suggest that miRNA-mediated translational control is derepressed following neuronal activation, consistent with activity’s effect on protein synthesis.

Results

Analysis of Hippocampal miRNA expression by Deep Sequencing

To understand the breadth of miRNA expression in the adult hippocampus, libraries from individual male C57BL/6 mice were constructed using standard protocols from Illumina and sequenced using an Illumina Genome Analyzer IIx. A total of 12 independently constructed libraries yielded an average of 9.85 x 10^6 reads per library resulting in a total of 118 million reads (Table 1). The reads were processed using the miR-Intess pipeline [13]. After removal of reads that had no or short insert sequences or lacked 3' adapter sequence, 70.8 million reads were accepted for further analysis. Of the accepted reads, 58% were mappable to the mouse genome (NCBI Mus musculus assembly 37, Table S1). As is typical of small RNA libraries, mappable reads contained a variety of small RNAs and fragments of abundant cellular RNA species (Table 1). Although varying between libraries, 84.1% of mappable reads were composed of known miRNAs (Table 1, Figure S1). Variability in library composition is likely due to a combination of biological and technical variation. The libraries used in this analysis were generated from hippocampi from mice either housed in their home cage or mice treated with electroconvulsive shock (ECS) as model of neuronal activity (see below). To establish a baseline for further comparison we examined the relative expression of miRNAs that expressed >0.1% of the total miRNA reads in libraries from mice not treated with ECS (Figure 1, black bars).

To directly compare the results from our libraries with a comparable data set, we plotted the percent reads from a mouse hippocampal miRNA library sequenced using the 454 platform [14]. Though similar in composition, there are differences in relative abundance of various miRNAs (Figure 1, gray bars). This is in agreement with a recent study that demonstrated that differences in library generation protocols and the sequencing platform used alter the absolute quantification of miRNA [15]. In total we identified 119 high confidence miRNAs previously described in miRBase release 15 (Table S2), although less than half of these miRNAs (53) are expressed at levels greater than 0.1% of total miRNA reads. The study by Pena et al. [14] concurs that 40/53 of these miRNAs are expressed at levels >0.1% of total

### Table 1. Composition of Small RNA libraries.

| Read Class          | No Treatment | Time post-ECS |
|---------------------|-------------|--------------|
|                     | NT-1        | NT-2         |
|                     | 0.5 h-1     | 0.5 h-2      |
| known miRNA         | 4874592     | 1183270      |
| candidate novel miRNA | 1859       | 913          |
| confident novel miRNA | 364       | 1205         |
| homolog known miRNA | 335        | 1082         |
| non-hairpin         | 91159       | 163027       |
| novel known miRNA   | 4651        | 4900         |
| other RNA           | 1877        | 5692         |
| other hairpins      | 118597      | 186996       |
| rRNA                | 598         | 2187         |
| repeats             | 110194      | 107996       |
| sncRNA              | 780         | 958          |
| senseRNA            | 9265        | 22459        |
| senseRNAAnc         | 6191        | 11026        |
| siRNA               | 2474        | 6328         |
| siRNAAnc            | 1501        | 3288         |
| snRNA               | 391         | 1368         |
| snoRNA              | 16408       | 16102        |
| tRNA                | 80817       | 288036       |

Read Class: Known miRNA: previously identified isoforms of known miRNA including star strands, candidate novel miRNAs: have no bad features and at least one positive or one negative feature and 2 or more positive features listed in results section, confident novel miRNA: have no negative features and at least two positive features listed in the results section, Homolog of known miRNA: known miRNA that contains sequence features that distinguish from miRBase sequence, novel known miRNA: reads that represent previously undocumented processing variants of known miRNA, other RNA: RNA reads that are inconsistent with miRNA features and are not members of the following classes of RNA: rRNA, tRNA, repeats (including repetitive elements), sense miRNA fragments, sense ncRNA, potential siRNAs, potential siRNAs derived from ncRNAs, snRNA, snoRNA, or tRNA. All miRNA star strands are ‘counted’ as a part of the mature miRNA’s category.

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Identification of Novel miRNAs

Deep sequencing of small RNA libraries is the established standard for identification of novel miRNAs. The miR-Intess data pipeline uses strict guidelines to identify novel miRNAs from deep sequencing data sets. In order to be considered a ‘confident’ novel miRNA, a sequence must have at least two of the following positive features: (1) the sequence is represented in multiple libraries. (2) The sequence maps to a genomic region containing a hairpin that is consistent with Dicer and Drosha processing. (3) The hairpin that the sequence lies within must be considered thermodynamically stable by Randfold. All novel miRNAs were required to have a sequenced star strand. The sequence is rejected as a novel miRNA if the mature sequence has any of the following negative features: the sequences is inconsistent with being a Dicer product, has a high degree of 5’ end variability, a hairpin sequence that is too short, if the sequence maps to greater than 10 locations in the genome, has high G-C content, if the sequence is not in 18–24 nt range, or if the read overlaps the predicted hairpin sequence. With these stringent requirements we identified 15 confident novel miRNAs (Table 2). The majority of the novel miRNAs have a sequenced star strand further bolstering the confidence in our assessment of these sequences as bona fide miRNAs. Using the Targetscan algorithm, we predicted targets of the novel miRNAs that have a novel seed sequence (Table S3). Though distributed throughout the genome, 3/15 of the novel miRNAs we identified lie with the introns of the serotonin receptor 2c (Htr2c) gene (Figure 2).

Neuronal activity regulates miRNA expression

Neuronal activity has profound effects on gene expression and mRNA translation. As miRNAs are intertwined with both of these processes, we investigated how miRNA expression is influenced by neuronal activity in vivo. Electroconvulsive shock (ECS) was chosen as a model for in vivo neuronal activity for several reasons, ECS generates a massive, synchronous depolarization of neurons in the CNS, thus permitting a time-course analysis of miRNA expression following activity. This technique faithfully recapitulates activity-dependent induction of the classic immediate early genes (IEGs) activity-regulated cytoskeletal protein (Arc) and FBJ osteosarcoma oncogene (Fos) (Figure 3A). Finally, ECS induces robust depolarization without significant tissue damage or cell death (Figure 3B). Following treatment with ECS, animals were sacrificed at 0.5, 1, 3, 6 and 24 h and RNA from the hippocampus was collected. These time points were selected to encompass changes in miRNA expression associated with IEG induction as well as expression of the plasticity-associated late response genes [16]. After sequencing one complete time course (designated with -1 suffix, Table 1), reproducibility of expression was determined by sequencing a biological replicate of the time course (designated with -2 suffix, Table 1). Though there are few studies that report replicates of small RNA deep sequencing, correlation between the untreated samples in both time courses was in line with those demonstrated in at least one other study [Pearson’s correlation, R² = 0.75, Figure 4A,[17]). However, by both absolute and relative read counts, the results of these two time courses were distinct (Table S1). Unsupervised hierarchical clustering revealed a pronounced ‘batch effect’ between time course replicates 1 and 2 (Figure 4B). The ‘batch effect’ observed in this case is observed by the tighter correlation in expression levels within replicates than between treatment groups. At the current time it is impossible to determine whether this effect is due to library construction or due to the sequencing process.

Given the ‘batch effect’ between replicates, it is unlikely that an accurate assessment of changes in miRNA expression level could be derived from this deep sequencing data. To more accurately characterize changes in miRNA expression, we performed an abbreviated time course analysis using the Taqman low-density array (TLDA) platform (Life Technologies) followed by extensive low-throughput quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Importantly, the rodent TLDA ‘A’ array includes assays for 46/53 miRNAs that are expressed at
levels greater at 0.1% of total miRNA (Figure 1). A cut-off Ct value of 32 cycles was used for analysis as this threshold yielded a Pearson’s R^2 correlation between technical replicates of 0.99 (Figure 4C). The TLDA platform necessarily relies on relative quantification of individual assays to a reference. Rather than relying on a single reference, we normalized expression levels to the geometric mean of five small noncoding RNAs (Rnu6, Snord65, Snord68, Snord87, and Rny1), an approach that generally yields a more stable reference. The results of the TLDA showed no signs of the ‘batch effect’ observed in the deep sequencing data. Rather, individual arrays grouped more closely by time-point than replicate, suggesting that biological instead of technical variation is observed in these data (Figure 4D).

TLDA analysis demonstrated that the majority of miRNAs’ levels respond to activity (Table S4). Unsupervised hierarchical clustering of expression profiles revealed that most miRNAs fell into three classes (Figure 5A, Figure S2). Class 1 (11.6% of profiled miRNA) is composed miRNAs that increase in response to ECS and includes the activity-regulated miRNA, miR-134[11]. Class 2 (14.6% of profiled miRNA) is composed of miRNA that are not strongly regulated up or down. Table 2.

| Designation   | Seed     | Family | chr | Start  | Stop   | Location     | reads | star | Conservation# |
|---------------|----------|--------|-----|--------|--------|--------------|-------|------|---------------|
| mmu-mir-1298  | UCAUUCG  | novel  | X   | 143499422 | 143499570 | Intronic: Htr2c | 13855 | yes  | m,r,h,p,ma    |
| mmu-mir-1264  | AAAUCUU  | novel  | X   | 143445144 | 143445231 | Intronic: Htr2c | 31    | yes  | m,r          |
| mmu-mir-1912  | ACAGAAC  | miR-410| chr7| 66828011  | 66828078  | Intronic: Htr2c | 667   | yes  | m,r,ma       |
| mmu-mir-344d-1| AUAAUAC  | miR-410| chr7| 66828011  | 66828078  | Intronic: Htr2c | 1853  | yes  | m            |
| mmu-mir-3106  | GCCACAU  | novel  | chr8| 16168742 | 16168851  | Intronic: Cim1d | 1973  | yes  | m,r,g        |
| mmu-mir-3106  | GCCACAU  | novel  | chr14| 31385335 | 31385394  | Intronic: Tkt | 103   | yes  | m            |
| mmu-mir-344d-3| AUAAUAC  | miR-410| chr7| 68871130  | 68871214  | Intronic: Htr2c | 1922  | yes  | m            |
| mmu-mir-3059  | UCCUCUCU | novel  | chr10| 101235313 | 101235419 | Intronic: Mga4   | 88    | yes  | m,p,g,d,r,ma |
| mmu-mir-3095  | GGACACU  | novel  | chr4 | 58453883 | 58453971  | Intronic: Lpar1  | 211   | yes  | m            |
| mmu-mir-344c  | GAUCUAG  | miR-344| chr7| 68982184  | 68982294  | Exonic: ncRNA   | 221   | yes  | m,r          |
| mmu-mir-344i  | AGUCAGG  | novel  | chr7| 69230109 | 69230196  | Intronic: Ptrim  | 12    | yes  | m,r          |
| mmu-mir-5709  | UACGCAC  | novel  | chr17| 67375487 | 67375575  | Intronic: Ptprm  | 31    | yes  | m            |
| mmu-mir-3093  | GUGGACA  | novel  | chr3 | 88019095 | 88019178  | Intronic: Htr2c  | 82    | yes  | m,h          |
| mmu-mir-3066  | UGGUGGC  | novel  | chr12| 17362189 | 17362288  | Intronic: Nol10  | 74    | yes  | m,p,d        |
| mmu-mir-5710  | CUUGGAA | novel  | chr9 | 54556120 | 54556188  | miRtron:Dnaj04  | 30    | yes  | m,r          |

*During the manuscript review process, some novel miRNAs were identified by other groups. #m: Mus musculus, r: Rattus norvegicus, h: Homo sapiens, p: Pan troglodytes, ma: Macaca mulatta, g: Gallus gallus, d: Danio rerio

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Figure 2. Genomic location of novel and previously identified miRNA in the serotonin receptor 2c (Htr2c) locus. (A) The gene models for identified transcripts at the mouse Htr2c locus. (B) Transcripts identified from the homologous locus in other mammalian species. (C) Conservation of genomic sequence in a multiple-species genomic alignment.

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down by activity at late timepoints. The largest class, Class 3 (73.4% of profiled miRNA), is composed of miRNAs that are in some cases initially induced by activity, but show a pronounced decrease in expression by 12 and 24 h post-ECS. The most abundant miRNA expressed in the hippocampus largely fell into Class 3 showing decreased expression at 12 and 24 h post-ECS (Figure 5B). The observed effect on miR-124 expression, a highly abundant neuronal miRNA, required NMDA receptor activation as the competitive NMDA receptor antagonist completely blocks the effect of ECS on the change in miR-124 expression (Figure 5C).

To validate these results and to increase the resolution of the time course of miRNA expression, we conducted a low-throughput analysis using qRT-PCR of select high and low abundance miRNAs (Figure 6). This is consistent with observations of rapid transcriptional induction of miRNA expression following neuronal activity ([10,11,12,13]). Though some individual qRT-PCR results show divergent result from TLDA analysis, the general trends born out in the array analysis hold true. Examples of both convergent and divergent qRT-PCR results are illustrated in Figure 6. In general, higher abundance miRNAs showed more concordant results (i.e. miR-124, miR-181a, miR-26a), while low abundance (miR-410) or miRNAs with multiple close-related family members (let-7i, miR-99b) showed more divergent results.

**Discussion**

This study defines the breadth of the hippocampal miRNA transcriptome, at the basal state and following robust synchronous neuronal activity. Using deep sequencing, we provide the most in-depth investigation of a single tissue’s miRNA expression published to-date. In total, we identified 15 novel miRNAs using a stringent set of criteria. Among the novel miRNAs identified, three lay within the introns of Htr2c, bringing the total of intronic miRNAs encoded the Htr2c locus to 7. Additionally, we identified three new members of the mir-410 family. Finally, using a variety of platforms we made the striking observation that the majority of miRNAs expressed in the hippocampus are down-regulated in response to robust neuronal activity at a relatively late time point. This finding in particular is important to how we think about the effects of activity on the programming of RISC and the regulation of target miRNAs.

We identified a large number of candidate novel miRNAs in our sequencing analysis (Table 1, 412 loci), among which are some bona fide miRNA. A recent study called into question nearly one third of the total miRNAs registered in miRBase 14.0 due to a lack of significant sequence support or absence of characteristic miRNA features [19]. Consistent with this observation, the majority of candidate miRNAs identified did not meet the strict criteria for novel miRNA discovery. However, 15 confident novel miRNAs were identified that pass a stringent set of criteria (see Results) that are compatible with criteria outlined in Chiang et al. (2010). Because of the depth of sequencing (118 million reads), we feel confident that we have identified all miRNAs expressed in the major cell types of the adult murine hippocampus.

Several interesting features are found among the novel miRNAs identified in this study. First, the majority of the miRNAs identified represent novel seed-sequence families, and therefore represent a host of previously unappreciated potential miRNA-target interactions. Using the TargetScan algorithm, we predicted potential target sites in mouse 3’ UTRs for all novel miRNAs with novel seed families (Table S4). In addition to novel seed families, 2/15 novel miRNAs share a seed sequence with mir-410. This expansion of the mir-410 family is interesting considering the potential targets of the mir-410 family include numerous gene of interest to the neuroscience community including ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 (ST8Sia4), cytoplasmic polyadenylation element binding protein 4 (Cpeb4), and phosphodiesterase 4B (Pde4b) among others. Second, though transcribed from a variety of genomic locations (introns, non-coding RNAs, mirtron, etc.) 3/15 novel miRNAs are embedded within introns of the serotonin receptor 2c (Htr2c) gene (Table 2, Figure 2). The introns of Htr2c contain previously annotated miRNAs: mir-764, mir-1912, mir-1264 in intron 2 and mir-446 in intron 4. The potential function of the novel miRNAs as well as the previously annotated miRNAs in the introns of Htr2c in serotonceptive...
neurons remains uninvestigated. Also uninvestigated is the possibility that a portion of the Htr2c knockout mouse’s phenotype could be attributable to the disruption of any of the 7 intronic miRNAs of that locus [20]. The Htr2c knockout mouse has a complicated phenotype that includes disruptions in appetite control, epilepsy, and diabetes [21]. It is unclear what the effects on the intronic miRNAs are in Htr2c knockout mouse as the targeting cassette disrupts exon 5, which lies downstream of all the intronic miRNAs. Third, 10/15 the novel miRNAs are conserved in one or more vertebrate species. This suggests that the majority of the novel miRNAs could be selected for during the course of vertebrate evolution, implying that they have a significant function.

Our investigation of activity-dependent regulation of miRNA expression began with analysis of deep sequencing libraries constructed from mice treated with ECS. Unsupervised hierarchical clustering revealed that the contents of libraries were highly dependent on the replicate, rather than on treatment with ECS (Figure 4B). This type of ‘batch effect’ is pervasive in a wide variety of high-throughput platforms including deep sequencing [22]. This may be due in part to library construction methodologies which introduce significant biases to the frequency of small RNA cloning [15]. However, the role of day-to-day variation in the operation of sequencers in the observed bias in the data cannot be ruled out. These factors may contribute to what is at least anecdotally observed as the poor correlation in results between miRNA expression profiling platforms [23]. Improvements in small RNA cloning methodologies, including the use of mutant T4 RNA ligases in association with 5′ adenylated adapters [24], will likely reduce bias and improve reproducibility in library construction. Our data suggest that even with improvements in cloning methodologies that multiple replicates of experimental samples are necessary to assess changes in miRNA expression in a quantitative manner, especially when fold changes in expression are relative modest (<2-fold) as in this data set.

To avoid the confounding effects observed in our deep sequencing, we turned to qRT-PCR, in either TLDA or single miRNA format, to analyze dynamic changes in miRNA expression level in response to neuronal activity. Most strikingly, the majority of miRNAs decreased in expression level at late time points following ECS (Figure 5, Table S4). Higher temporal resolution analysis showed that a fraction of the miRNAs undergo rapid induction in expression, but then rapidly decrease in expression, while other miRNA undergo no induction and simply decrease in expression. This early phase following activity may reflect direct transcriptional induction of miRNA gene expression or increased miRNA processing of existing transcripts (Figure 6). However, the majority of miRNA transcripts decrease in abundance following this initial phase of induction. This is consistent with a recent study that demonstrated that neuronal miRNAs exhibit an unusually high rate of turnover, and that this turnover is accelerated by glutamatergic activity [25]. These researchers demonstrated that under the conditions of transcriptional inhibition that neuronal miRNAs decay rapidly. In our
study we observe that the same phenomenon likely exists in the intact hippocampus in the absence of pharmacological inhibition of transcription. Together with another report of activity-dependent degradation of miRNAs in Aplysia suggests that these are evolutionarily conserved phenomena [26]. Our observations (Figure 5) are consistent with either an accelerated turnover of mature miRNA or the global repression of miRNA biogenesis. In either case, the widespread decline in mature miRNA will create a permissive environment for translation, with implications for translation-dependent processes such as learning and memory where precise control of protein levels are critical. To this point, a recent report of a conditional deletion of Dicer in the forebrain of young adult mice showed that during a period shortly after deletion, the Dicer mutants showed improved performance in memory tasks [27]. During this period of improved memory after Dicer deletion, the levels of residual miRNA expression are similar to those found in Class 3 of ECS-treated mice (Figure 5A). These observations suggest that the 50–70% reduction in miRNA expression observed in Class 3 miRNA may have a physiological impact on learning and memory.

Activity-dependent regulation of miRNA expression was first described in the context of the CREB-responsive miRNA miR-132 [10]. This study and subsequent miR-132-focused studies demonstrated activity dependent upregulation of miR-132 following sustained treatment of primary neuronal cultures with BDNF, KCl, or Bicuculline [28,29]. Similar activity-dependent induction of miRNA expression were recently described for the mir-379–mir-410 locus [11]. In agreement with these findings, we observe a number of miRNAs are induced immediately following brief, synchronous induction of activity (Figure 6). However, overall this elevation in miRNA expression is followed by a global decline in miRNA levels in most cases. A minority of miRNAs (Class 1, Figure 5A) do show extended periods of increase following ECS. The differences in results between the current study and in vitro models is likely due to the mechanism of activity induction. Two studies recently demonstrated that miR-132 is transcriptionally induced in vivo in response to activity [12,18]. As in our study, these studies observed induction of miRNA expression following activity, but both previous studies did not measure expression levels beyond 2 hours post-treatment.

An interesting consequence of the broad change in miRNA expression is the change in the spectrum of RISC-bound miRNA. It is generally believed that the number of Argonaute molecules, the miRNA-binding component of RISC, is the limiting factor for the amount of miRNA in a cell. If activity in general decreases the total amount of miRNA, then presumably there will be a ‘race’ to fill available Argonaute molecules. As miRNAs repopulate Argonaute proteins, the homeostatic ratio of various miRNA species may be altered, thus altering the translational profile of the neuron. It is interesting to consider that disorders associated with altered patterns of neuronal activity, such as schizophrenia and autism, may have an impact on the composition of neuronal miRNA expression solely through this type of activity-dependent mechanism. Consistent with this idea, a survey of miRNA expression in post-mortem tissue from autistic individuals revealed heterogeneous changes in miRNA expression [30].

![Figure 5. Expression profiling of hippocampal miRNA expression following electroconvulsive shock (ECS).](image-url)
geneity in expression of miRNAs in these individuals may be due to heterogeneity in patterns of neuronal activity. The role of changes in miRNA expression profiles in activity-dependent processes will certainly be the focus of future investigations.

Methods

Animals, RNA Isolation, and Histology

All animals were housed, cared for, and experiments conducted in accordance with the Johns Hopkins University Animal Care and Use Committee (Assurance #A3272-01) guidelines as specifically approved as a part of animal protocol # MO08M522. All experiments described in this study were specifically approved as a part of the aforementioned animal protocol. Mice were treated with electroconvulsive shock (ECS) using an ECT Unit (Ugo Basile) with following settings: 1 sec shock, 100 pulses/sec, 0.4 ms pulse width, 22 mA current. To test the requirement of NMDA receptor activation, mice were injected with either PBS alone or CPP (10 mg/ml i.p.) 1 hour prior to treatment with ECS. Following ECS, total hippocampal RNA was isolated from 6–8 week old C57BL/6 males using Trizol (Life Technologies) following the manufacturer’s protocol. RNA quality was assessed by electrophoresis and spectrophotometric analysis using a Nano drop spectrophotometer. For histological analysis, animals were first anesthetized using pentobarbital and perfused with 4% paraformaldehyde followed by cryoprotection in sucrose and cryosectioning. Sections were Nissl stained using standard methods and light micrographs capture using an Axioplan 2 (Zeiss).

Deep Sequencing

Libraries were constructed using the Illumina Small RNA Digital Gene Expression kit following the manufacturer’s protocol. RNA quality was assessed by gel electrophoresis and analysis using a Nanodrop spectrophotometer. In brief, 10 μg of total RNA was separated by denaturing gel electrophoresis, stained with ethidium bromide, and the fragment containing ~18–25 nt small RNAs was recovered. Following elution, the 5’ adapter was ligated to the small RNA using T4 RNA ligase. Following ligation, the products of this reaction were separated by denaturing gel electrophoresis and the band in the 40–60 nt range was excised and again eluted from the gel fragment. The 3’ adapter was then ligated using T4 RNA ligase to the eluted fragments followed by separation by denaturing gel electrophoresis. The 70–90 nt range was excised from the gel, eluted, and precipitated. The 5’ and 3’ adapter adapted small RNA was then reverse transcribed and subjected to PCR amplification using the Phusion HF (Finzymes) polymerase. Sequencing was conducted using an Illumina Genome Analyzer Ix at the UCLA Genome Sequencing Center. The miR-Intess pipeline used to process the sequencing output is described in detail elsewhere [13,31]. The raw sequence data is available through NCBI Gene Expression Omnibus (GSE32053).

TLDA and qRT-PCR

RNA isolated from the hippocampus was treated with RNase-free DNase I (Ambion) to remove contaminating DNA. For TLDA analysis, 750 ng of total RNA was used for reverse transcription using the Megaplex Pool A primer set (Life Technologies) and the MultiScribe RT system (Life Technologies).

Following loading, the TLDA mouse miRNA ‘A’ array was run on an ABI 7900HT Real-time PCR System (Life Technologies). Changes in miRNA expression were quantified using the \( \Delta \Delta C_t \) method normalizing the to geometric mean of the Ct values of 5 non-coding RNAs Rnu6, Snood65, Snood68, Snood87, and Ray1. Unsupervised hierarchical clustering was performed using Cluster 3.0 for the Macintosh and visualized using TreeView 1.1.3 for Macintosh OS X.

For low-throughput qRT-PCR, 500 ng of total RNA was reverse transcribed with the miScript RT System (Qiagen), allowing the assessment of both miRNA and mRNA expression. Expression of miRNAs was measured using pre-designed miScript primer sets (Qiagen). Real-time PCR analysis was conducted on an ABI 7900HT using Power SYBR-Green reagents (Qiagen). Changes in expression levels of transcripts were determined using the \( \Delta \Delta C_t \) method normalizing to Rn6 or Rp2. All assays were performed on RNA from 3–4 mice. All PCRs were performed in quadruplicate.

Statistics

All statistical analyses were performed using Prism (Graphpad) or Excel (Microsoft).

Supporting Information

Figure S1 Percent composition of individual libraries in this study. Each class of RNA is represented by a different color (Legend, right). (TIF)

Table S1 Summary of the mapping of reads. “Non-matching” reads were unable to be mapped to genome following removal of the adapter sequences. “Trimmed 3’ end” are reads that matched to the genome after trimming all but the 20 nt of the 5’ end of the read. “Perfect” refers to reads that match perfectly in the genome following removal of adapter sequence. (XLS)

Table S2 Summary of miRNA reads from all deep sequencing libraries. (XLS)

Table S3 Predicted target sites for novel miRNAs described in this study. (XLS)

Table S4 Fold changes in miRNA expression of all TLDA experiments for miRNAs with that amplified with a Ct value greater than 32. (XLS)

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Author Contributions
Conceived and designed the experiments: SME VLD TMD. Performed the experiments: SME MJK. Analyzed the data: SME EB. Contributed reagents/materials/analysis tools: EB SME. Wrote the paper: SME. Revised the manuscript: SME VLD TMD.

References
1. Costa-Mattioli M, Sossin WS, Klann E, Sonenberg N (2009) Translational control of long-lasting synaptic plasticity and memory. Neuron 61: 10–26.
2. Bassell GJ, Warren ST (2008) Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron 60: 201–214.
3. Kellleher RJ, 3rd, Bear MF (2008) The autistic neuron: troubled translation? Cell 135: 401–406.
4. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9: 102–114.
5. Schrott GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, et al. (2006) A brain-specific microRNA regulates dendritic spine development. Nature 439: 283–289.
6. Ishizuka A, Siomi MC, Siomi H (2002) A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev 16: 2497–2508.
7. Jin P, Zarnescu DC, Ceman S, Nakamoto M, Moswry J, et al. (2004) Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. Nat Neurosci 7: 113–117.
8. Edbaer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, et al. (2010) Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron 63: 373–384.
9. Stark KL, Xu B, Bagchi A, Lai WS, Lai H, et al. (2008) Altered brain microRNA biogenesis contributes to phenotypic deficits in a 22q11-deletion mouse model. Nat Genet 40: 751–760.
10. Vo N, Klein ME, Varlamova O, Keller DM, Yamamoto T, et al. (2005) A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. Proc Natl Acad Sci U S A 102: 16426–16431.
11. Fiore R, Khadayberdin S, Christensen M, Siegel G, Flavell SW, et al. (2009) A new regulatory mechanism in mammalian neurons. J Neurogenetics 9: 153–161.
12. Wibrand K, Panja D, Tiron A, Ofte ML, Skaftnesmo KO, et al. (2010) Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. Cell 141: 618–631.
13. Rajasekharapathy P, Finnara F, Sheridan R, Sander C, et al. (2005) Identification of microRNAs of the herpesvirus family. Nat Methods 2: 269–276.
14. Krej J, Bauskamp V, Markiewicz I, Stadler MB, Ribi S, et al. (2010) Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. Cell 141: 618–631.
15. Konopka W, Kiryk A, Novak M, Herwerth M, Parkitna JR, et al. (2010) MicroRNA loss enhances learning and memory in mice. The Journal of Neuroscience: the official journal of the Society for Neuroscience 30: 14835–14842.
16. Klein ME, Lioy DT, Ma I, Impye S, Mandel G, et al. (2007) Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci 10: 1513–1514.
17. Fehniger TA, Wylie T, Germino G, Leong JW, Magrini VJ, et al. (2010) Next-generation sequencing identifies the natural killer cell microRNA transcriptome. Genome Res 20: 1590–1604.
18. Nadelman AS, DiRocco DP, Lambert TJ, Garelick MG, Le J, et al. (2010) Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. Hippocampus 20: 492–498.
19. Chiang HR, Schoenfeld LJ, Ruby JG, Auyeung VC, Spies N, et al. (2010) Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. Genes Dev 24: 992–1009.
20. Osoke M, Neu R, Loeb GB, McManus MT (2008) Unintentional miRNA ablation is a risk factor in gene knockout studies: a short report. PLoS Genet 4: e34.
21. Tecott LH, Sun LM, Akana SF, Strack AM, Lowenstein DH, et al. (1995) Eating disorder and epilepsy in mice lacking 5-HT2c serotonin receptors. Nature 374: 542–546.
22. Leek JT, Scharpf RB, Bravo HC, Simcha D, Langmead B, et al. (2010) Tackling the widespread and critical impact of batch effects in high-throughput data. Nat Rev Genet 11: 733–739.
23. Baker M (2010) MicroRNA profiling: separating signal from noise. Nat Methods 7: 687–692.
24. Pfeffer S, Sewer A, Lagos-Quintana M, Sheridan R, Sander C, et al. (2005) Identification of microRNAs of the herpesvirus family. Nat Methods 2: 269–276.
25. Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Palmanovica J, et al. (2009) Characterization of small RNAs in aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. Neuro 63: 803–817.
26. Konopka W, Kiryk A, Novak M, Herwerth M, Parkitna JR, et al. (2010) MicroRNA loss enhances learning and memory in mice. The Journal of Neuroscience: the official journal of the Society for Neuroscience 30: 14835–14842.
27. Klein ME, Lioy DT, Ma I, Impye S, Mandel G, et al. (2007) Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci 10: 1513–1514.
28. Wayman GA, Davace M, Ando H, Forin D, Varlamova O, et al. (2008) An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. Proc Natl Acad Sci U S A 105: 9093–9098.
29. Abu-Elneel K, Liu T, Gazzaniga FS, Nishimura Y, Wall DP, et al. (2008) An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. Proc Natl Acad Sci U S A 105: 9093–9098.
30. Nudelman AS, DiRocco DP, Lambert TJ, Garelick MG, Le J, et al. (2010) Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. Hippocampus 20: 492–498.
31. Berezikov E, Thuemmler F, van Laake LW, Kondova I, Bontrop R, et al. (2006) A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev 16: 2497–2508.