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Characterization of Small RNAs in *Aplysia* Reveals a Role for miR-124 in Constraining Synaptic Plasticity through CREB

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SUMMARY

Memory storage and memory-related synaptic plasticity rely on precise spatiotemporal regulation of gene expression. To explore the role of small regulatory RNAs in learning-related synaptic plasticity, we carried out massive parallel sequencing to profile the small RNAs of *Aplysia californica*. We identified 170 distinct miRNAs, 13 of which were novel and specific to *Aplysia*. Nine miRNAs were brain enriched, and several of these were rapidly downregulated by transient exposure to serotonin, a modulatory neurotransmitter released during learning. Further characterization of the brain-enriched miRNAs revealed that miR-124, the most abundant and well-conserved brain-specific miRNA, was exclusively present presynaptically in a sensory-motor synapse where it constrains serotonin-induced synaptic facilitation through regulation of the transcriptional factor CREB. We therefore present direct evidence that a modulatory neurotransmitter important for learning can regulate the levels of small RNAs and present a role for miR-124 in long-term plasticity of synapses in the mature nervous system.

INTRODUCTION

miRNAs are a class of conserved, 20–23 nucleotide (nt) noncoding RNAs that depend on the RNAi machinery for maturation and function and are able to mediate cleavage or translational repression of their target mRNAs by preferentially binding to their 3′UTRs (Filipowicz et al., 2008; Bartel, 2009). Discovery of the first miRNAs in *C. elegans* led to an understanding of their regulatory role in cell lineage specification (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000). The subsequent development of methods for the large-scale identification of miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) and the resulting functional studies revealed that miRNAs control many other cellular functions, including proliferation, metabolism, apoptosis, immunity, and, more recently, neuronal growth and plasticity.

To obtain a more complete inventory of small RNAs that may have a role in learning-related synaptic plasticity, we used a large-scale identification approach to mine and functionally screen the small RNAs of the marine snail *Aplysia*. The large, stereotyped, easily identifiable neurons of *Aplysia* make it a practical choice for studying the distribution and function of small RNAs at the resolution of single cells and single synapses. Critical components of the neural circuitry of the *Aplysia* gill-withdrawal reflex can be reconstituted in cell culture, allowing for a well-controlled study of axonal growth, synapse formation, stabilization, and synaptic plasticity. The evolutionary closeness of *Aplysia* to vertebrates and mammals also makes *Aplysia* generally attractive as a model system for addressing the function of small RNAs (Moroz et al., 2006).

In *Aplysia*, both short-term memory lasting minutes and long-term memory lasting days have been well characterized in the gill-withdrawal reflex in response to sensitization, a simple form of learned fear (reviewed in Kandel, 2001). In an *Aplysia* sensory-motor culture system (Montarolo et al., 1986), delivery of one pulse of serotonin (5HT), a modulatory neurotransmitter released in the intact animal by sensitizing stimuli, elicits PKA-dependent short-term facilitation lasting minutes. By contrast, five spaced pulses of serotonin cause both PKA and MAPK to translocate to the nucleus (Martin et al., 1997b), thereby releasing inhibition by the repressor CREB2 and activating CREB-dependent transcription, leading to long-term synaptic facilitation and growth of new synaptic connections. Thus, in sensitization, as in many other forms of learning, nuclear activation of CREB is an important component of a general switch that converts short-term into long-term plasticity in both vertebrates and invertebrates (Dash et al., 1990; Barco et al., 2002). In

addition, studies on both the gill-withdrawal reflex and the mammalian hippocampus has delineated the importance of local protein synthesis at the synapse in sustaining synapse activity independent from the distant cell body (reviewed by Sutton and Schuman, 2006; Martin and Zukin, 2006). Indeed, communication between the nucleus and the synapse, via the shuttling of mRNA and proteins by kinesin motors, serves as still another critical regulatory point in the induction of long-term facilitation (Puthanveettil et al., 2008).

Since the spatiotemporal regulation of learning-related synaptic plasticity is extensive and complex, miRNAs appear well-suited to serve as negative regulators. The ability of miRNAs to selectively (Farh et al., 2005) and reversibly (Bhattacharyya et al., 2006) silence mRNAs allows for precise control, possibly in a combinatorial fashion, of relevant subsets of the mRNA population recruited during plasticity. Moreover, their ability to form autoregulatory loops (Rybak et al., 2008; Johnston and Hobert, 2003) suggests their potential involvement in either homeostatic or switch-like events during various phases of synaptic plasticity, an inherently multistable phenomenon. Several studies have demonstrated the involvement of brain-specific miRNAs in synapse formation and of miRNA ribonucleoprotein complexes (miRNPs) in controlling local protein synthesis associated with stable memory (reviewed in Schratt, 2009). These findings have encouraged us to explore systematically the miRNA population of the *Aplysia* central nervous system to understand their functions during learning-related synaptic plasticity.

We identified small RNAs in neuronal and nonneuronal cell populations in *Aplysia* that allowed the identification of brain-enriched miRNAs. We have functionally characterized the most highly abundant, well-conserved, brain-specific miRNA: *Aplysia* miR-124. This miRNA is specific to the presynaptic sensory neuron where it is rapidly downregulated by serotonin. In the absence of serotonin regulation, miR-124 provides an inhibitory constraint on synaptic plasticity and long-term facilitation through the regulation of CREB, the transcriptional switch critical for converting short- to long-term facilitation.

RESULTS

Aplysia miRNAs and Their Evolutionary Context

We prepared small RNA cDNA libraries from isolated central nervous system (CNS) and from the whole animal with CNS removed. Within the CNS, we also generated small RNA libraries from dissected abdominal and pleural ganglia. The libraries from the whole animals and CNS were sequenced using 454 sequencing technology yielding a total of about 250,000 sequence-reads for each library. The abdominal and pleural libraries were sequenced by traditional Sanger sequencing until ~2000 reads were collected for each library.

Because we lacked an assembled genome, we first built an *Aplysia*-specific annotation database to distinguish miRNAs from turnover of abundant and conserved noncoding RNAs such as rRNAs, tRNAs, or snRNAs. The total content of rRNAs, tRNAs, and snRNAs taken together varied between 5% and 25% per library (Table S1). To be considered a miRNA, the residual sequences needed to satisfy the following three criteria. (1) Precise 5′ end processing: length variants of members of a sequence family preferentially aligned to the 5′ end. (2) Fold-back precursor structure: a fold-back structure had to be identified comprising a genomic fragment retrieved from unassembled trace archives. (3) Cloning of the miR* sequence: since double-stranded miR/miR* processing intermediates are assembled in an asymmetric fashion, capture of miR* that generates short 3′ overhangs when paired to the mature miRNA is further evidence for prototypical miRNA biogenesis.

The *Aplysia* genome trace sequence archives do not yet cover the full *Aplysia* genome. We therefore considered certain clone sequences that did not map to trace sequences as miRNAs, if we could map them to miRNA precursors annotated in other species. We identified 170 distinct miRNAs in *Aplysia*, of which 157 were orthologs to known miRNAs in other species and 13 were specific to *Aplysia*. The new discoveries are cataloged in Table 1. All miRNAs are cataloged in Table S2. Sixty sequences that were abundantly cloned and that demonstrated good 5′ processing but that were neither conserved nor mapped were designated miRNA candidates (Table S3). The evidence for each miRNA is summarized in Table S4. The overall abundance of miRNAs in the small RNA libraries ranged from 50% to 80%, consistent with the miRNA content in small RNA libraries prepared from other species (Aravin et al., 2003; Landgraf et al., 2007).

A phylogenetic analysis of the *Aplysia* transcriptome revealed that *Aplysia* is closer in evolutionary distance to the vertebrates than are *C. elegans* and *D. melanogaster* (Moroz et al., 2006). We similarly find that *Aplysia* miRNAs more closely resemble vertebrate miRNAs both in sequence similarity of individual genes and in the abundance of shared miRNA genes. We grouped the 170 distinct *Aplysia* miRNAs into 103 miRNA gene families based primarily on seed sequence similarity (Table S5), of which 41 families are conserved specifically in vertebrates, whereas only 13 map specifically to invertebrates (Figure 1A). When we fit the observed miRNA gene gains and losses onto various phylogenetic trees, we find that our data best fit a model where *Aplysia* is a very ancient ancestor to the invertebrates, lies outside the *D. melanogaster/C. elegans* clade, and more directly straddles the invertebrate and vertebrate lineages (Figure 1B). The orthology relationships used to construct the phylogeny are given in Table S6. A salient feature in support of this model is the presence of 46 miRNAs that are preserved from vertebrates to *Aplysia* but subsequently lost in *D. melanogaster* and *C. elegans* (Figure 1B). Taken together, these findings illustrate that *Aplysia* miRNAs are ancient and well conserved, with relatively few losses or gains of genes, which makes it a distinctive model organism among invertebrates that shares important genomic similarities with vertebrates and mammals.

We observe one striking aspect of evolutionary history regarding the miR-9/79 gene family. The invertebrate-specific miR-79 and the vertebrate- and *Drosophila*-specific miR-9 are expressed in equal proportions in *Aplysia* and are in fact star sequences of each other, found on opposite strands of the same precursor hairpin (Figure 1C). It is likely, then, that *Aplysia* mir-9/79 is a single gene that displays symmetric maturation patterns for both strands, whereas in other species, the gene has duplicated to give rise to multiple gene copies with asymmetric strand preference producing either miR-79 in other invertebrates or miR-9 in vertebrates.

Table 1. New miRNAs Discovered in *A. californica*

Mature miRNA Name	Mature miRNA Sequence	Number of Reads	Star Sequence Cloned
miR-100001	UGCCAUUUUUAUCAGUCACUGUG	17379	+
miR-100053	UGCCCUAUCCGUCAGGAACUGU	2169	+
miR-100097	UCAGCAGUUGUACCACUGAUUUGA	634	+
miR-100098	UGAGACAGUGUGUCCUCCCUUG	493	–
miR-100102-5p	AUUUGGCACUUGUGGAAUAAUCG	285	+
miR-100106	CAUCUACCUAUCCUUCUUCUUC	221	–
miR-100060	CUUGGCACUGGCGGAAUAGUCAC	174	–
miR-100102-3p	AUUAUACACCGGUGCCAAAU	151	+
miR-100072	UUACCCUGGAGAACCGAGCGUGU	125	–
miR-100070	GAAGCGGGUGCUCUUAUUU	109	–
miR-100090	UAUCCGCUCACAAUUCCCC	102	–
miR-100087	UUGUGACCGUUAUAAUGGGCAUU	75	–
miR-100091	AGCGGUGAUAUUUUUGUCUGGC	69	–

All sequences shown here had a mapping to the genome with a recognizable precursor hairpin structure and good 5′ processing of mature sequence length variants. The predominantly cloned sequence is given along with the number of sequence reads, including its predominant length variants and an indication of whether the star sequence was (+) or was not (–) cloned.

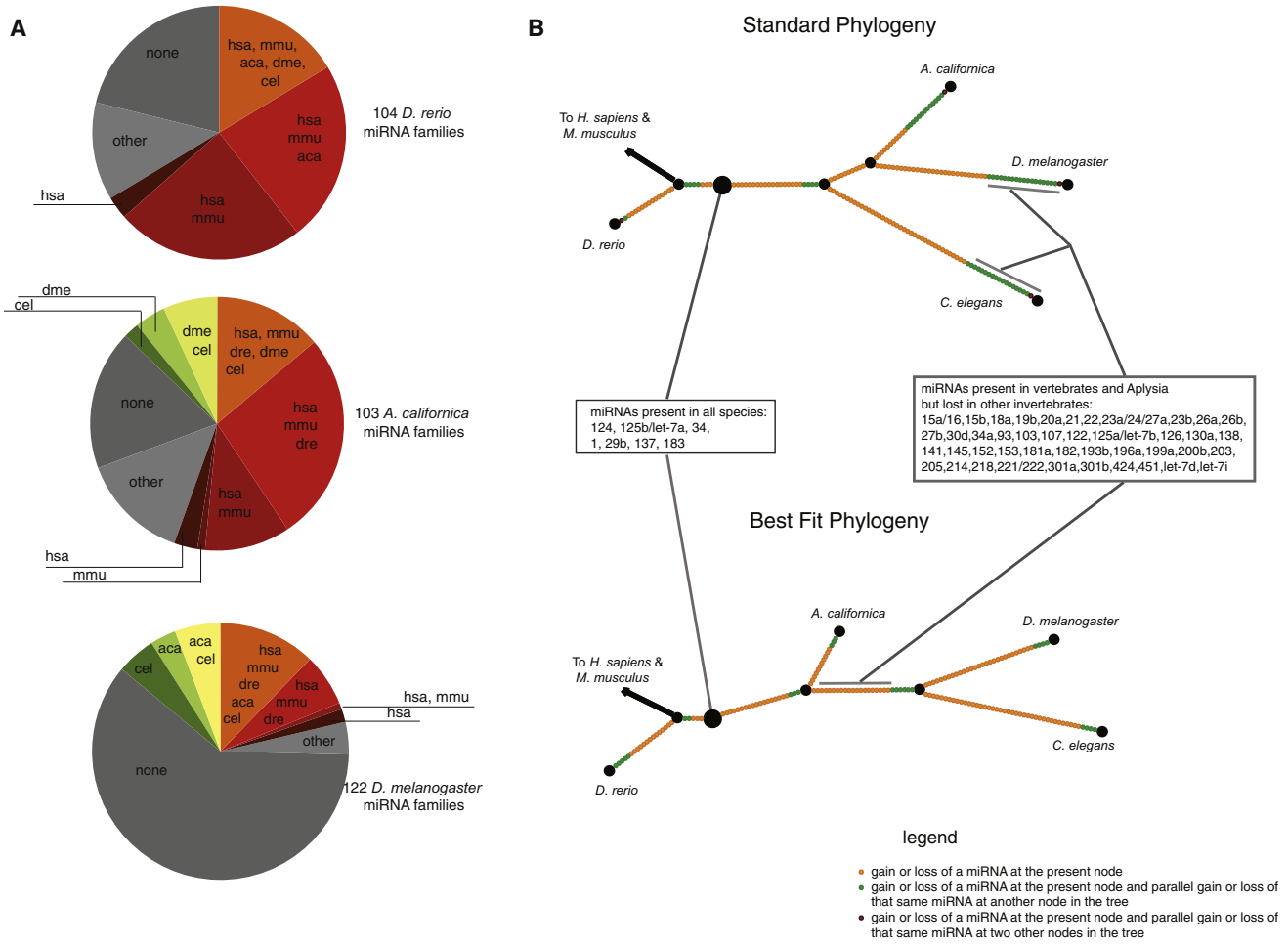
CNS-Enriched miRNAs and Their Cellular and Subcellular Distribution

Deep sequencing revealed the expression of over 100 distinct miRNA genes expressed in the *Aplysia* CNS. The miRNAs comprising the top 95% of clones are shown in order of their abundance in Figure 2 with their enrichment in the CNS versus the rest of the body. Nine miRNAs are either brain specific or brain enriched by cloning (Figure 2, and confirmed with northern blotting in Figure 3A), three of which are miRNAs unique to *Aplysia.* In general, there was good overlap of the miRNAs of *Aplysia* CNS with the miRNAs of the human and rodent brain, but notable exceptions include the high abundance and brain enrichment of miR-22c (Figure 3A), miR-184 (Figure 3A), miR-34b, and miR-190, where studies in other species have not found CNS enrichment for these miRNAs (Chen et al., 2005; Ruby et al., 2007; Landgraf et al., 2007). The multicopy cistrons of miR-1/133a and miR-206/133b, which are muscle specific in vertebrates and *D. melanogaster*, were abundantly expressed in *Aplysia* CNS. Finally, the low expression of miR-9 and the complete absence of miR-128 in *Aplysia* CNS are noteworthy because both are highly abundant and brain-specific in vertebrates.

To learn which miRNAs might function in a compartment-specific manner, we developed a protocol (described in Experimental Procedures) for in situ hybridization of miRNAs in *Aplysia* using synthetic DNA probes. We dissected a functional circuit containing a sensory and motor neuron from *Aplysia* ganglia, placed them in coculture, and examined the in situ hybridization patterns for various miRNAs (Table 2). We found that miR-124 stained much more intensely in the sensory neuron compared with the motor neuron (Figure 3B), and a 4 nt mismatch containing control probe showed no signal. We also consistently observed both a perinuclear density for mir-124, as well as punctuate staining in the processes (Figure 3C). Further in situ hybridization studies of the more abundant miRNAs in CNS revealed several other miRNAs (such as miRs 22c, 125c, let-7a, 100, and 8b) that were specifically expressed in the sensory neuron compared to the motor neuron (Figure 3B) and some that were enriched in either the cell body alone (miR-1) or in the neurite processes alone (miR-100001) (Figure 3C). The differential expression of miRNAs between sensory and motor neurons is also apparent from miRNA clone frequencies of abdominal versus pleural ganglia, the latter of which contain many more sensory neurons.

miRNAs Can Be Regulated by 5HT, a Modulatory Neurotransmitter Important for Learning

miR-124 has been shown to be important during neuronal differentiation and in specifying neuronal identity (Lim et al., 2005; Makeyev et al., 2007; Visvanathan et al., 2007; Cheng et al., 2009). Our finding that miR-124 is relatively absent in the motor neuron of a sensory-motor coculture gave us the first indication that miR-124 may not be present in all neurons and may have functions in addition to maintaining neural identity. We therefore asked: might miR-124 be regulated by synaptic activity? Specifically, we wanted to know whether it might be modulated by serotonin, a neurotransmitter important for learning. We found, by northern analysis, that already within 1 hr of exposure to five spaced pulses of serotonin the levels of miR-124 were consistently reduced by 2-fold (Figure 4A). These findings were corroborated by in situ hybridization analysis, which also showed a drop in miR-124 levels in both the sensory neuron cell body and neurite processes within 1 hr after washout from five pulses of serotonin (Figure 4B). No change in miR-124 levels was observed in cells treated with just one pulse of serotonin (Figure S1). To determine how long it takes for miR-124 levels to return to baseline after exposure to five pulses of 5HT, we performed a time course analysis and found that miR-124 levels continue to drop even 2 hr after 5HT treatment, but then slowly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103 A. californica miRNA families
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B
Standard Phylogeny
A. californica
To H. sapiens & M. musculus
D. melanogaster
D. rerio
C. elegans
miRNAs present in all species: 124, 125b/let-7a, 34, 1, 29b, 137, 183
miRNAs present in vertebrates and Aplysia but lost in other invertebrates: 15a/16,15b,18a,19b,20a,21,22,23a/24/27a,23b,26a,26b, 27b,30d,34a,93,103,107,122,125a/let-7b,126,130a,138, 141,145,152,153,181a,182,193b,196a,199a,200b,203, 205,214,218,221/222,301a,301b,424,451,let-7d,let-7i
Best Fit Phylogeny
A. californica
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To H. sapiens & M. musculus
C. elegans
D. rerio
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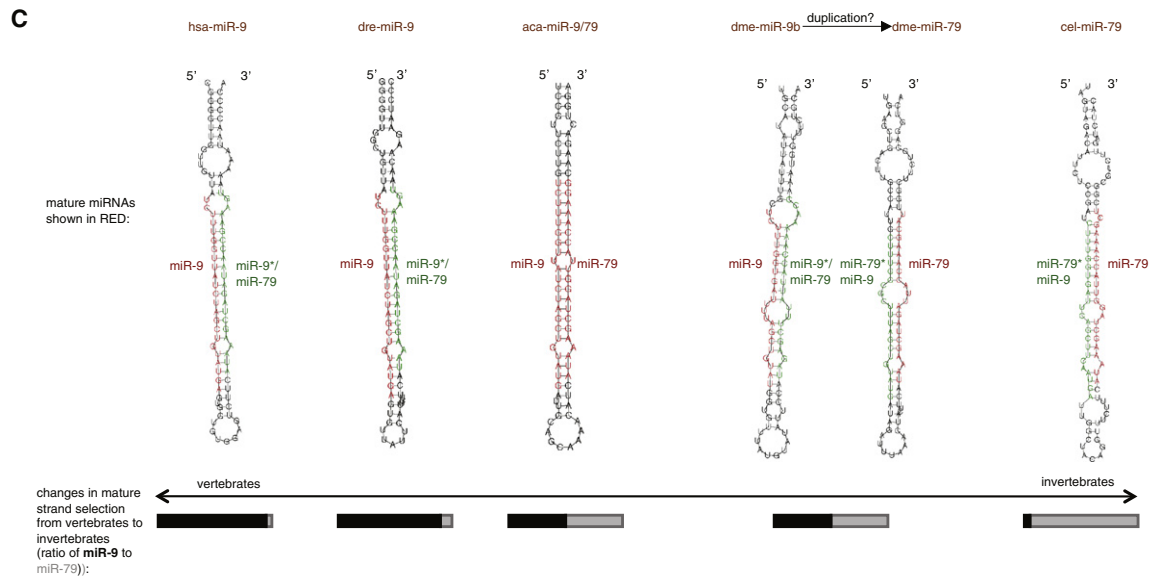
C
hsa-miR-9
dre-miR-9
aca-miR-9/79
dme-miR-9b
duplication?
dme-miR-79
cel-miR-79
5' 3'
mature miRNAs shown in RED:
miR-9
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miR-9*
changes in mature strand selection from vertebrates to invertebrates (ratio of miR-9 to miR-79):
vertebrates
invertebrates

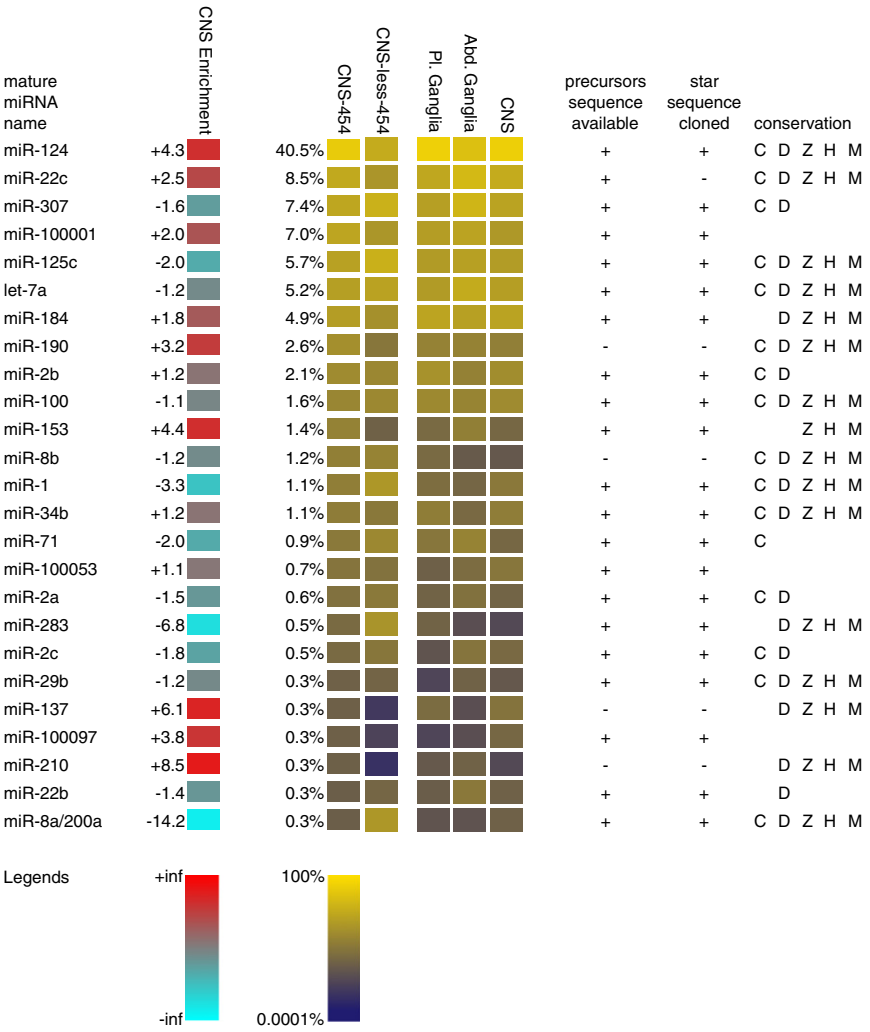


Figure 2. The Abundant miRNAs Observed in *A. californica* CNS

The top 95% of miRNA clone content from the CNS library is shown, along with enrichment in the brain as compared to the whole animal, distribution in abdominal and pleural ganglia, the existence or absence of a precursor in the genome together with cloning evidence for its star sequence, and finally homology relationships to *H. sapiens* (*H*), *M. musculus* (*M*), *D. rerio* (*Z*), *D. melanogaster* (*D*), *and C. elegans* (*C*).

reaccumulate, returning to baseline by 12 hr (Figure 4C). To better understand the mechanism underlying the serotonin-induced regulation of miR-124, we tested whether the miR-124 precursor levels were also affected by 5HT and found by real-time PCR that pre-miR-124 levels remained unaffected in sensory neurons after exposure to five pulses of 5HT (Figure 4D). This indicated that the regulation of miR-124 occurs downstream to the biogenesis of the precursor species, either at the level of the RNase III Drosha processing or turnover of the Argonaute-bound miRNA complex. Since 5HT is known to activate several downstream signaling pathways, including PKA (Castellucci et al., 1980), MAPK (Martin et al., 1997), PKC (Sacktor and Schwartz, 1990), and the proteasome (Hegde et al., 1997), we applied inhibitors of each of these molecules, in the presence of 5HT, to determine which, if any, contributes most to the regulation of miR-124. We found, by northern analysis, that a MAPK inhibitor (U0126) almost fully abolished the 5HT-induced downregulation of miR-124. By contrast, inhibitors of PKC (bisindolylmaleimide) and the proteasome (MG-132) had no effect, and a PKA inhibitor (KT5720) showed a modest but insignificant attenuation of the 5HT-induced miR-124 effect (Figure 4E).

In screening other miRNAs for serotonin-dependent regulation, we found one miRNA (miR-184) that had an even more pronounced (3-fold) reduction and others that either showed modest (miR-125c) or no (miR-2b) regulation by serotonin (Figure 4A). We therefore demonstrate that a synaptic neurotransmitter can regulate miRNA levels. In the case of miR-124, we find that this occurs rapidly, is sustained for many hours, occurs through MAPK signaling, and affects only the mature miRNA levels, not the precursor form. The ability of serotonin to modulate miR-124 levels is of specific interest because its previously known function in neuronal differentiation suggested constitutive expression in mature neurons to maintain neuronal identity.

Figure 1. *Aplysia* miRNAs Are More Similar to Those of Vertebrates than Invertebrates

(A) Pie charts illustrating the fraction of miRNA families in a given species that bear homology relationships with miRNA families in other species. Homology with vertebrates is displayed in shades of red, invertebrates in yellow and green. Absence of conservation, or mixed conservation patterns (other) are displayed in shades of gray.

(B) The evolutionary relationship of the miRNAs in five species, as understood through gain and loss events, is mapped onto both a standard phylogeny (based on rRNA distances) and an alternate phylogeny (based on best fit of our data). In both cases, *Aplysia* is closer to the vertebrates than is *D. melanogaster* or *C. elegans*. The alternate tree, however, is able to minimize the number of parallel and independent evolutionary events (green and red beads) that must occur to satisfy the phylogenetic architecture. We highlight the eight miRNAs that are present in all represented species and the 46 miRNAs that were preserved from vertebrates to *Aplysia* but subsequently lost in other invertebrates.

(C) miR-9 and miR-79, although thought to be distinct miRNAs emerging from separate loci, are in fact star sequences of each other. Here we show that miR-9 is preferentially expressed in vertebrates, while miR-79 is preferentially expressed in invertebrates. *Aplysia*, however, expresses both in equal proportions in 3-p/5-p fashion.

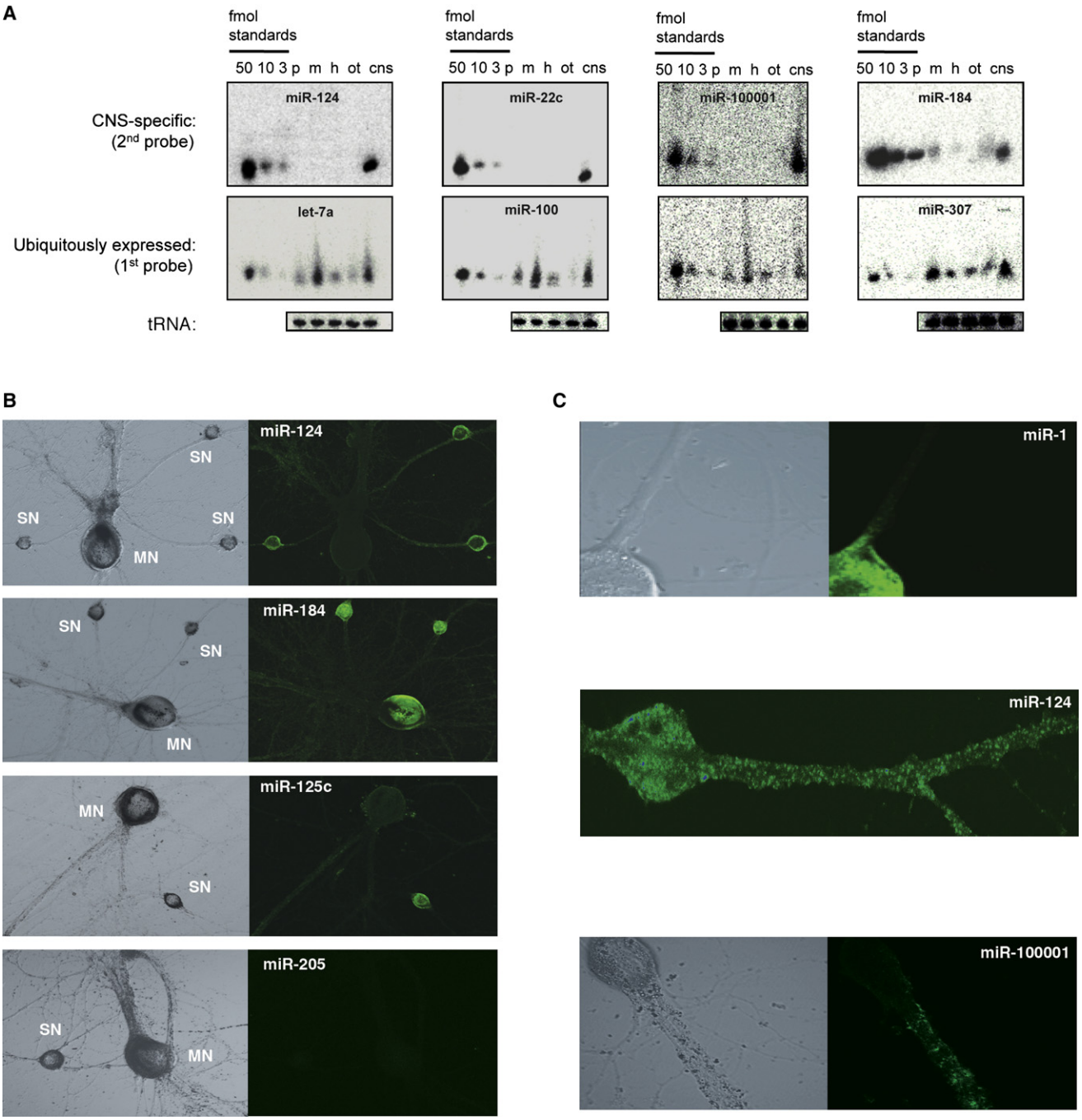


Figure 3. Characterization of miRNA Tissue and Cell Specificity and Subcellular Distribution

(A) Northern blots showing the expression of eight different mature miRNAs across various tissues including (p) hepatopancreas, (m) muscle, (h) heart, (ot) ovotestis, and (cns) central nervous system. 20 μg of total RNA was loaded in each lane (except in the case of miR-100001, where 40 μg of total RNA was loaded due to difficulty detecting signal). Detection of synthetic miRNAs loaded on the far left of the blots at a concentration of 50 fmol, 10 fmol, and 3 fmol serve as positive controls. tRNA bands are shown to control for equal loading of samples.

(B) Projection images of 10× confocally acquired images from 1 μm slices through a sensory (SN)-motor (MN) coculture in situ hybridized with DNA probes complementary to the mature miRNA sequence. As a negative control, some cells were probed for mir-205, which is not expressed in *Aplysia* neurons, and therefore show no staining. miR-124 and miR-125c are exclusively found in the sensory neuron (SN).

(C) Projection images of 40× confocally acquired images showing an example of a miRNA that is primarily found in the cell body (miR-1), primarily in the cell process (miR-100001), and in both compartments (miR-124).

Table 2. Summary of miRNA Expression Patterns in an *Aplysia* Sensory-Motor Coculture as Assessed by in Situ Hybridization

	Number of Reads (CNS)	CNS Enrichment	Sensory Neuron	Motor Neuron	Cell Body	Cell Process
miR-124	88,678	++	+		+	+
miR-307	28,561		+	+		+
miR-125c	24,803		+		+	+
miR-22c	20,405	+	+		+	+
let-7a	17,452		+		+	+
miR-184	12,629	+	+	+	+	+
miR-100001	8,690	+	+	+	+	+
miR-1	6,593		+	+	+	
miR-2b	6,128		+	+	+	+
miR-100	5,339		+		+	+
miR-8b	4,040		+		+	+
miR-153	2,951	++	+	+	+	+

For each miRNA, the table indicates whether it was enriched (++), present (+), or absent () in the cell types and compartments listed. The table is sorted by miRNAs of decreasing abundance in the CNS, and their clone frequencies are listed.

miR-124 Provides an Inhibitory Constraint on Long-Term Facilitation through the Regulation of CREB-Dependent Transcription

To determine the physiological relevance of the 5-HT-induced changes in miR-124 levels, we altered miR-124 levels in sensory neurons and analyzed the effect on the 5-HT-induced long-term facilitation (LTF) of the synapses between the sensory and motor neuron (Figures 5A and 5B). Injection of a duplex miR-124 *mimic* (Dharmacon, Inc.), designed to increase the levels of miR-124 in sensory neurons, caused a significant reduction in LTF as measured at 24 and 48 hr after exposure to five pulses of 5-HT ($n = 10$) when compared to uninjected controls in the same coculture ($n = 9$, $F_{(1,17)} = 5.27$, $p < 0.05$, two-way ANOVA with one repeated-measure [time]; Figures 5D and 5E). Conversely, injection of the single-stranded antisense miR-124 *inhibitor* (Dharmacon, Inc.), designed to reduce the levels of miR-124, caused a significant increase in synaptic facilitation of the 5HT-treated synapses ($n = 14$) with respect to untreated controls ($n = 14$) as measured at 24 and 48 hr ($F_{(1,26)} = 4.70$, $p < 0.04$, two-way ANOVA with one repeated-measure; Figures 5J and 5K). Control experiments with the injection of scrambled miR *mimic* ($n = 16$) and scrambled miR *inhibitor* ($n = 16$) did not show significant changes in LTF ($p > 0.05$, two-way ANOVA with one repeated measure; Figures 5G, 5H, 5M, and 5N). The observed differences in LTF among the different treatments were not due to differences in the basal strength of the synaptic connections in the different experimental groups (Figures 5F, 5I, 5L, and 5O). In situ hybridization confirmed that the miR-124 *mimics* and *inhibitors* were able to increase or decrease, respectively, the levels of miR-124 in sensory neurons (Figure 5C).

To further support these observations, we also performed physiological experiments using an alternative knockdown method. To inhibit miR-124, we bath-applied antisense 2′-O-methyl-oligoribonucleotides conjugated with the peptide penetratin (Qbiogene, Inc.). The penetratin-conjugated inhibitor (200 nM) is capable of crossing the membrane of cultured *Aplysia* neurons and of inducing inhibition of miR-124, as determined by in situ hybridization (Figure 5P, also shown for inhibition in whole ganglia by northern blotting in Figure 6A). To induce a significant inhibition, we incubated cells with the penetratin-conjugated inhibitor for 24 hr before testing the basal amplitude of the EPSP and applying five pulses of 5-HT. The experiments with penetratin-conjugated miR-124 inhibitor confirmed that LTF was significantly enhanced following inhibition of miR-124 ($+127.1 \pm 16.36$, $n = 9$) as compared to controls treated with penetratin-conjugated to a miR-194 inhibitor ($+67.35 \pm 18.18$, $n = 9$, $p < 0.01$, Newman-Keuls post-hoc test after two-way ANOVA; Figure 5R). The inhibition of miR-124, within these temporal limits, did not affect basal synaptic transmission while interfering with 5HT induced LTF (Figure 5Q).

How does the downregulation of miR-124 lead to long-term facilitation? To find potential targets of miR-124, we screened many genes relevant to plasticity and known to be regulated by serotonin for increases in expression levels after inhibition of miR-124. Desheathed pleural ganglia were incubated in antisense 2′-O-methyl-oligoribonucleotides conjugated with penetratin to inhibit miR-124 (confirmed by northern blot Figure 6A), following which total protein was extracted and western blotted. We found that inhibition of miR-124 led to a robust upregulation in the *Aplysia* pleural ganglia of CREB1, the activator of transcription required for long-term facilitation (Figure 6B). This is consistent with the fact that not only the protein (Bartsch et al., 1998) but also the CREB1 mRNA levels are upregulated by serotonin (Liu et al., 2008b). We also found several genes whose expression levels were unaffected by miR-124 inhibition (Figure 6B). Specifically, CREB2, the repressor that antagonizes CREB1 in synaptic depression, was unaffected by miR-124.

To be certain that miR-124 acts through CREB, we asked whether inhibition of miR-124 might affect the regulation of genes downstream to CREB. We observed that all three known immediate-response genes, induced by serotonin in a CREB-dependent manner, increased in their level of protein (Figure 6B) and in their level of transcript (Figure 6D) after inhibition of miR-124. These three genes are (1) ubiquitin C-terminal hydrolase (UCH) (Hegde et al., 1997), (2) CAAT enhancer binding protein (C/EBP) (Alberini et al., 1994), and (3) kinesin heavy chain (KHC) (Puthanveettil et al., 2008). The increased protein and mRNA levels of these three genes were specific, because control antisense inhibitor did not alter levels of UCH, C/EBP, or KHC, and moreover, inhibition of miR-124 did not affect other plasticity-related genes, such as MAPK, neurexin, and tubulin (Figures 6B and 6D). The observed induction of protein levels of UCH, C/EBP, and KHC by inhibition of miR-124 was further enhanced by exposure to 5HT (Figure S2). This suggests that the miRNA inhibition is just one of perhaps many parallel 5HT-mediated events that converge to activate CREB and its immediate-early genes.

A conserved putative target site (Figure S3) for miR-124 in the CREB1 3′UTR of vertebrates and mammals indicated that miR-124 might directly bind and inhibit the translation of CREB1 mRNA. To determine whether miR-124 directly binds and

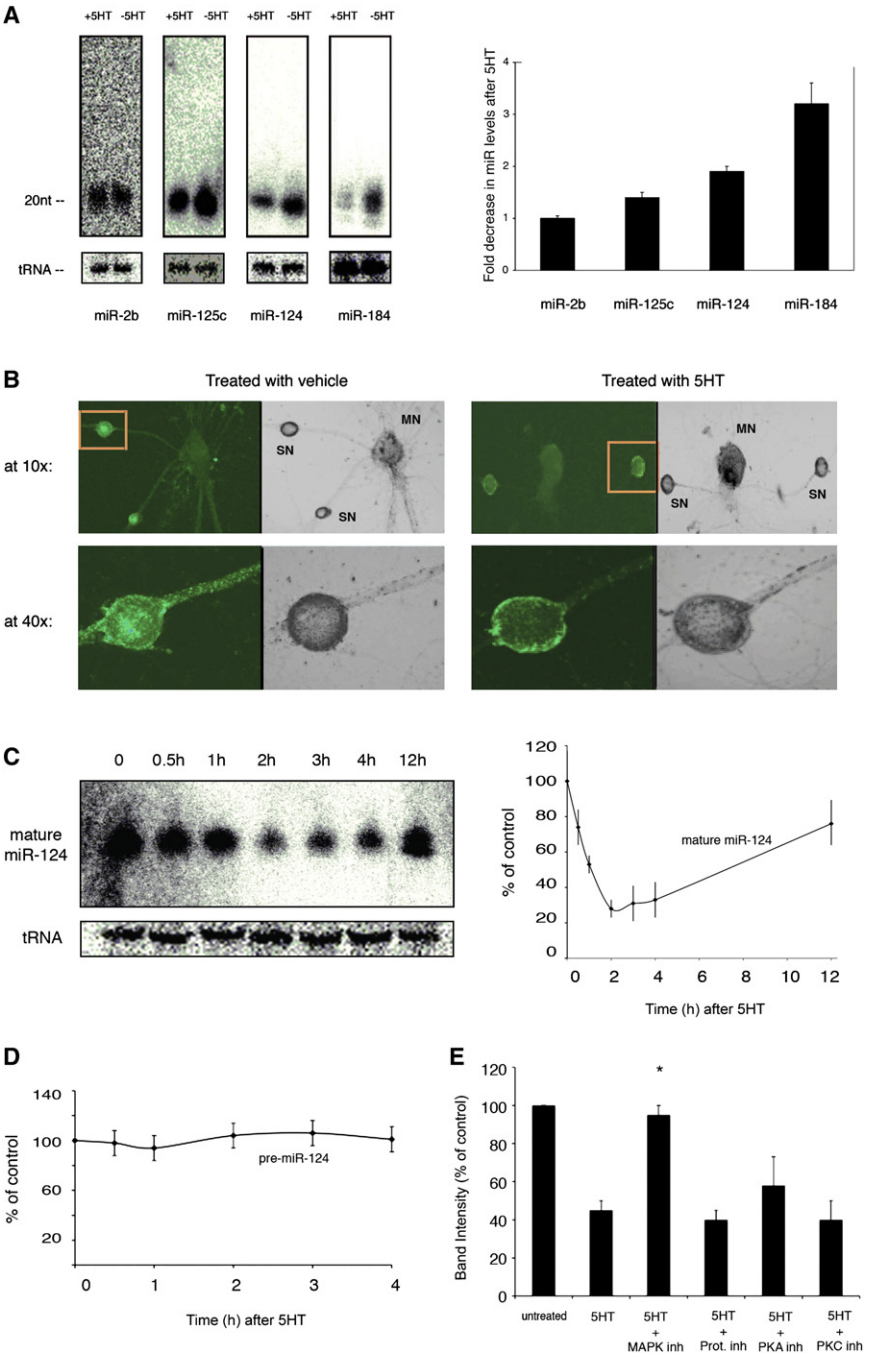


Figure 4. miRNAs Are Rapidly Downregulated by Serotonin

(A) Northern blot showing mature miRNA levels in untreated CNS (−5HT) and CNS treated with five spaced pulses of serotonin (+5HT). Blots were reprobed for tRNA to monitor equal loading of samples. Changes in miRNA levels are quantified and presented as a mean of six independent trails ±SD.

(B) In situ hybridization experiments in sensory-motor cocultures show that exposure to five pulses of 5HT causes a significant reduction of miR-124 levels in sensory neurons.

(C) Northern blot showing mature miRNA levels in CNS in control cells (0) and in CNS at 30 min (0.5 hr), 1, 2, 3, 4, and 12 hr after treatment with 5HT. The blots were reprobed for tRNA to control for equal loading of samples. The data are quantified in the right panel and presented as a mean of four independent trials ±SD.

(D) Real-time PCR data showing miR-124 precursor levels at 0, 0.5, 1, 2, and 4 hr after treatment with five pulses of 5HT. Data are shown as a mean of six independent trials ±SD.

(E) CNS were treated with 10 μM, in L-15, of each of the indicated inhibitors for 30 min prior to treatment with five pulses of 5HT. Following 1.5 hr after washout from 5HT and the inhibitors, total RNA was extracted, northern blotted, and probed for miR-124. Levels of miR-124 are given as mean band intensity from northern blots, and the data are presented here as a mean of four independent trials ±SD.

regulates *Aplysia* CREB, we cloned the full-length 3′UTR of *Aplysia* CREB1 and found a putative miR-124 binding site (9-mer seed + GU Wobble) near the poly A signal (Figure 6C). To test whether this site is functional, we examined the effect of miR-124 overexpression on a luciferase reporter fused to the CREB1 3′UTR. We found that miR-124 overexpression was able to repress the expression of the luciferase reporter by 45% ($p < 0.01$) when carrying the wild-type UTR but had no significant effect on the reporter when the seed of the miR-124 binding site in the UTR was disrupted by a 2 nt mismatch or when the reporter was fused to a truncated form of the UTR that no longer contained the miR-124 binding site (Figure 6C). In addition, the overexpression of an unrelated miRNA, let-7, had no significant effect on the reporter construct carrying the full-length CREB1 UTR (Figure 6C). As a positive control, an siRNA targeting the luciferase gene was able to repress luciferase activity by 80% (Figure 6C). These data indicate that miR-124 directly regulates *Aplysia* CREB1 by binding to its UTR near the poly A signal.

While cloning the *Aplysia* CREB1 3′UTR, we uncovered an isoform of CREB in *Aplysia*, which differs from the canonical CREB1 in its last exon and 3′UTR (Figure S5). This CREB isoform (which we term CREB1d) also bore a putative miR-124 target site but showed no direct regulation by miR-124 on luciferase reporter assays (Figure S4). The lack of regulation could be because the seed of this site is weak (six-mer seed + GU wobble) or because this site is in the ORF, which is considered to be functionally weaker than sites in the UTR (Bartel, 2009).

CREB1 is a transcription factor that acts as a switch to convert short-term, protein-synthesis-independent facilitation (requiring

one pulse of 5HT) into long-term, protein-synthesis-dependent facilitation (requiring five pulses of 5HT). Therefore, neurons that overexpress CREB1 require only one pulse, rather than five pulses, of 5HT for the induction of LTF (Bartsch et al., 1998). If CREB1 were indeed regulated in vivo by miR-124, the inhibition of miR-124 in sensory neurons, through its enhancement of CREB1, should require fewer pulses of 5HT to cause LTF. Indeed, in response to even a single pulse of 5-HT, cells treated with miR-124 inhibitor showed a significant level of facilitation after 24 hr (+42.66 ± 6.18, n = 35; comparable to that observed with CREB overexpression in Bartsch et al., 1998) with respect to a control miR-194 inhibitor (+20.57 ± 6.37, n = 22, $p < 0.04$, Newman-Keuls post-hoc test after two-way ANOVA; Figure 6E) and with respect to control cells treated with vehicle alone (+11.77 ± 8.18, n = 12, $p < 0.01$, Newman-Keuls post-hoc test after one-way ANOVA). The observed differences in the facilitation between treated and untreated groups were not due to differences in the basal strength of the synaptic connections as tested before 5HT application (Figure 6E). Together with the previous observations, these data support the idea that the 5-HT-dependent downregulation of miR-124, by allowing an increase in the levels of CREB and CREB-dependent transcription, is an important component of a switch that converts short-term to long-term synaptic plasticity.

DISCUSSION

In mining the miRNAs of *Aplysia* by deep sequencing, we have obtained what is perhaps the most comprehensive catalog of the miRNA population in a central nervous system. The well-conserved nature of these miRNAs encourages their study in other nervous systems. In *Aplysia*, it specifically allows study of miRNAs at the level of single cells and single synapses in processes ranging from neuronal development to synapse formation, stabilization, and plasticity. As more organisms are mined for their miRNAs, we are likely to gain a better understanding of how ancient and diverse miRNA gene families are and what constraints they face during evolution.

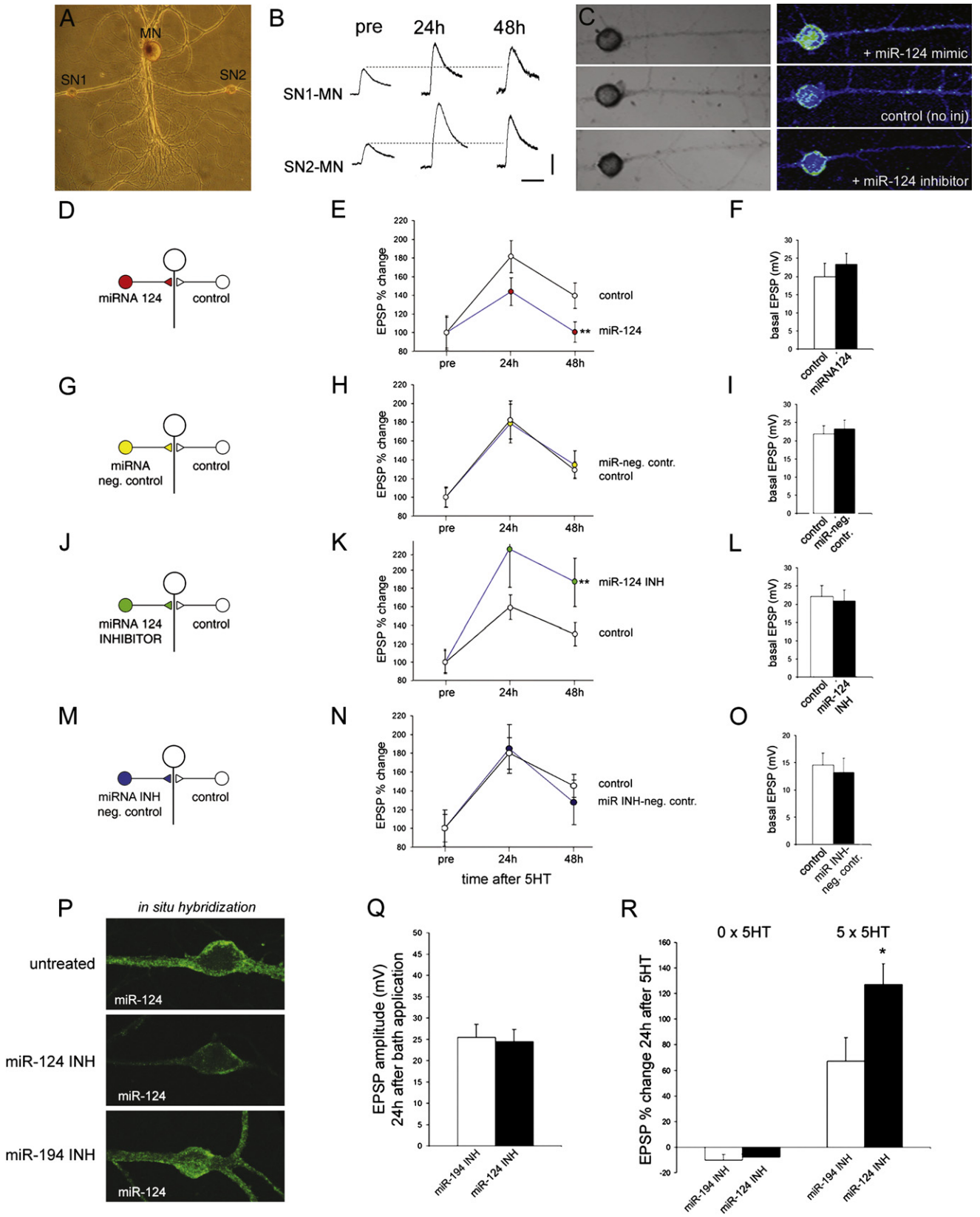
In this initial study, we describe 170 distinct miRNAs present in *Aplysia*, of which 13 were previously unknown. Recent studies (Lu et al., 2008; Liu et al., 2008a; Grimson et al., 2008) indicate that miRNA evolution has been dynamic and that most species have undergone dramatic changes in their miRNA gene content characterized by greater than normal rates of gene loss, gain, and duplication events. However, we find the *Aplysia* miRNA content to be particularly stable relative to its last common ancestor. *Aplysia* has gained only 13 miRNAs from its shared ancestry with vertebrates (though this number is likely to increase as the *Aplysia* genome coverage improves) and preserves over 40 miRNAs that are subsequently lost in *D. melanogaster* and *C. elegans*. Interestingly, the abundant miRNAs in *Aplysia* CNS appear to be as well conserved in invertebrates as vertebrates (Figure 2), whereas the whole animal miRNA population in *Aplysia* (Figure 1B) has a significant enrichment of shared vertebrate miRNAs, compared with invertebrate miRNAs, and many of these, such as miR-15/16, miR-145, and miR-221, are abundant and have important function in mammals. The similarity of genes between *Aplysia* and vertebrate systems is not entirely due to loss of genes in *C. elegans* and *D. melanogaster*. An analysis of well-conserved miRNAs shows that the vertebrate homolog is often more similar in sequence identity to the *Aplysia* homolog than it is to the homologs of *C. elegans* and *D. melanogaster*. The underlying similarity between *Aplysia* miRNAs and vertebrate miRNAs may also correlate with similarity in targets and in function, therefore strengthening the ability to use *Aplysia* as a model to understand the role of miRNAs in mammalian and even human neural function.

Rapid Serotonin Regulation of miRNAs Extends the Scope of Neurotransmitter Function

Expression analysis in cultured neurons of sensorimotor synapses revealed several miRNAs that were localized to distinct cells and subcellular compartments. Of the miRNAs that were screened, the striking enrichment of miR-124 in the sensory neuron with relative absence of expression in the motor neuron was most surprising. Earlier studies of miR-124 found that it had a ubiquitous and constitutive expression pattern in most neuronal cell types of the mammalian brain, which together with its lack of expression in progenitor cells, suggested a primary role for miR-124 in specifying and maintaining neuronal cell identity. Our studies of miR-124 in *Aplysia* revealed that, in addition to being nonuniformly expressed in adult neurons, miR-124 is rapidly and robustly regulated by the neurotransmitter serotonin, indicating additional roles for miR-124 in mature neurons. Several other miRNAs showed a similar downregulation by serotonin, suggesting a general mechanism by which synaptic activity might relieve negative constraints on gene expression during learning-related plasticity.

Our finding that small RNAs can be regulated by conventional neurotransmitters extends further the scope of neurotransmitter actions. Neurotransmitters were first appreciated in the context of their ability to (1) regulate gating of ion channels and subsequently to (2) covalently modify protein substrates by activating second messenger pathways. Subsequently, it was found that transmitters (3) also regulate transcription (reviewed in Kandel, 2001). We now describe a fourth function of neurotransmitter action: the regulation of small RNAs. These considerations raise the further question: how are the miRNAs regulated by serotonin? Recent studies have uncovered two major stages of regulation during miRNA biogenesis: one at the Drosha cleavage step that converts the primary transcript into a miRNA precursor and the second at the Dicer cleavage step that converts the precursor to the mature form (Obernosterer et al., 2006; Heo et al., 2008; Michlewski et al., 2008; Viswanathan et al., 2008). The ability of serotonin to selectively affect mature miR-124 levels, without affecting its precursor, argues for regulation during Dicer processing or during RISC incorporation and stabilization by Argonaute or even perhaps is the result of passive turnover of the miRNA in response to increased turnover of their target mRNAs.

The study by Ashraf et al. (2006) was the first to show learning-dependent changes in RISC and that this was dependent on the proteasome. In light of their finding, we reasoned that the proteasome may also be involved in the serotonin regulation of miR-124, especially since the changes in miRNA levels are rapid and may mean enhanced degradation rather than impeded


A
MN
SN1
SN2
B
pre
24h
48h
SN1-MN
SN2-MN
C
+ miR-124 mimic
control (no inj)
+ miR-124 inhibitor
D
miRNA 124
control
E
EPSP % change
control
miR-124
**
pre
24h
48h
F
basal EPSP (mV)
control
miRNA124
G
miRNA
neg. control
control
H
EPSP % change
miR-neg. contr.
control
I
basal EPSP (mV)
control
miR-neg. contr.
J
miRNA 124
INHIBITOR
control
K
EPSP % change
miR-124 INH
control
L
basal EPSP (mV)
control
miR-124 INH
M
miRNA INH
neg. control
control
N
EPSP % change
control
miR INH-neg. contr.
time after 5HT
O
basal EPSP (mV)
control
miR INH-neg. contr.
P
in situ hybridization
untreated
miR-124
miR-124 INH
miR-124
miR-194 INH
miR-124
Q
EPSP amplitude (mV)
24h after bath application
miR-194 INH
miR-124 INH
R
0 x 5HT
5 x 5HT
EPSP % change 24h after 5HT
*
miR-194 INH
miR-124 INH
miR-194 INH
miR-124 INH

maturation. However, we found that inhibiting the proteasome had no effect on the serotonin-induced downregulation of miR-124. Instead, we did observe that a MAPK inhibitor almost fully abolished the ability of serotonin to regulate miR-124. MAPK is one of the major signaling molecules downstream of serotonin that is known to activate CREB (Martin et al., 1997; Impey et al., 1998), and our data would suggest that one way it does so is by relieving miR-124 inhibition of CREB. The implication of MAPK in miR-124 regulation is of further interest because a GO term analysis of potential neuronal miR-124 targets (Table S7) indicates that several of these genes are MAPK regulated. The dissection of the precise mechanism by which MAPK downregulates miR-124 will require first an understanding of the MAPK substrates in the RNAi pathway and then an exploration of how phosphorylation events, say on Dicer or Argonaute, may lead to the destabilization of the mature miRNAs.

miR-124 Acts through CREB to Constrain Long-Term Plasticity

We find that miR-124 serves as a negative constraint on serotonin-induced long-term facilitation, since increased or decreased miR-124 levels in sensory neurons leads to a significant inhibition or enhancement, respectively, of synaptic facilitation. In particular, the inhibition of miR-124 confers to sensorimotor synapses a greater sensitivity for serotonin, since just one pulse of serotonin is sufficient to cause long-term facilitation. These physiology data also suggest that miR-124 inhibition is just one of many 5HT-mediated events that activate CREB to induce long-term facilitation, since the inhibition of miR-124 alone, in the absence of 5HT, does not lead to long-term facilitation. Therefore, while the observed effects of the miR-124 manipulations on LTF are of a significant magnitude, it is likely that these effects would be even greater if there were a coordinated manipulation of several miRNAs that act together in parallel pathways during synaptic plasticity. The observation that miR-124 levels affect facilitation both at 24 and 48 hr after exposure to spaced pulses of serotonin suggests that miR-124 regulation is required not only for the induction phase but that it is also critical for the maintenance phase of synaptic facilitation. Since miR-124 levels return back to baseline within 12 hr after exposure to serotonin, the initial drop in miR-124 during this time window appears to be sufficient enough to upregulate the relevant transcripts to allow for facilitation for up to 48 hr after exposure to serotonin. Indeed, the upregulation of many plasticity-related transcripts are transient and fall into this initial time window. The data also suggest that miR-124 does not significantly affect or contribute to serotonin-independent processes such as basal and constitutive synaptic activity. How-ever, since all of our experiments were conducted on several-day-old cultures, at which point the cells and synapses are fully mature and stable, our studies leave open the possibility that miR-124 contributes to serotonin-independent processes in immature neurons such as neurite out-growth and synapse formation.

The negative constraint that miR-124 imposes on synaptic facilitation is mediated, at least in part, by its direct regulation of CREB. The fact that miR-124 inhibition significantly and specifically increases CREB1 levels, along with immediate downstream genes such as UCH, C/EBP, and KHC, that miR-124 serotonin kinetics parallels the CREB1 serotonin kinetics, and that miR-124 inhibition can provide the switch necessary to convert short-term facilitation into long-term facilitation all strongly support the conclusion that miR-124 can tightly control CREB and CREB-mediated signaling during plasticity. CREB has been extensively studied over the years for its regulation by kinase-dependent posttranslational modifications, such as phosphorylation by PKA and MAPK. The present study, however, is one of the first to address posttranscriptional regulation of CREB. While this additional level of regulation might appear redundant, for example by paralleling the function of CREB2, it is likely that miR-124 inhibition allows for more rapid and transient control over CREB expression, as well as the opportunity for CREB to be drawn into various distinct downstream

Figure 5. miR-124 Negatively Constrains Serotonin-Dependent Long-Term Heterosynaptic Facilitation

(A) Phase contrast micrograph of the experimental model used for the electrophysiological experiments. An *Aplysia* L7 motor neuron (MN) is contacted by two sensory neurons (SN1 and SN2).

(B) Representative current-clamp electrophysiological recordings of EPSPs in motor neurons after extracellular stimulation of the sensory neuron. For each individual sensorimotor synapse, EPSPs were recorded before as well as 24 and 48 hr after 5 × 5HT treatment.

(C) Phase contrast (left column) and fluorescent micrographs (right columns) of in situ *hybridized* miR-124 levels in cultured sensory neurons that were injected with either the miR-124 *mimic* (upper row), *inhibitor* (lower row), or left uninjected (middle row).

(D, G, J, and M) Schematic representation of the different treatments that were applied to the sensorimotor cocultures for electrophysiological experiments. In each coculture, one of the two sensory neurons was injected either with 5 μM miR-124 *mimic* (D), miR *mimic* negative control (G), miR-124 *inhibitor* (J), or miR *inhibitor* negative control (M), whereas the other sensory neuron was left untreated as a control.

(E, H, K, and N) Graphs reporting the percentage change in EPSP amplitude measured at 24 hr and 48 hr after 5 × 5HT application with respect to pretreatment values in the different experimental groups. Percent changes in EPSP are shown as an average of 10 (E), 16 (H), 14 (K), and 16 (N) independent trials ±SD.

(F, I, L, and O) Bar graphs showing the average amplitude of EPSPs measured at synapses formed by SN1 and SN2 before injection and 5HT treatment in the different experimental groups to control for any change in basal synaptic strength that might contribute to observed changes in LTF. Basal EPSP amplitudes are shown as an average of 10 (E), 16 (H), 14 (K), and 16 (N) independent trials ±SD.

(P) An alternate miR-124 inhibition method was employed. Sensory neurons treated anywhere from 4 hr to 24 hr with 2-O-methyl oligonucleotides antisense to miR-124 conjugated to penetratin show a significant reduction in endogenous miR-124 levels, as compared with untreated cells, or cells treated with a control 2′O-methyl oligonucleotide antisense to miR-194 also conjugated to penetratin.

(Q) Bar graph showing the mean amplitude of EPSPs measured at cultured sensorimotor synapses 24 hr after the bath application of either a penetratin-conjugated miR-124 inhibitor (200 nM) or a control penetratin-conjugated miR-194. The bath application the miR-124 inhibitor does not significantly alter the average basal synaptic strength of sensorimotor synapses as compared with controls. Basal EPSP amplitudes are shown as an average of nine independent trials ±SD.

(R) Bar graph showing the average percentage synaptic facilitation measured at 24 hr after treatment with either zero or five serotonin pulses in cultures that had been preincubated with either the penetratin-conjugated miR-124 inhibitor or the control penetratin-conjugated miR-194 inhibitor. The miR-124 downregulation enhances facilitation but only in the presence of 5HT. Percent changes in EPSP are shown as an average of nine independent trials ±SD.

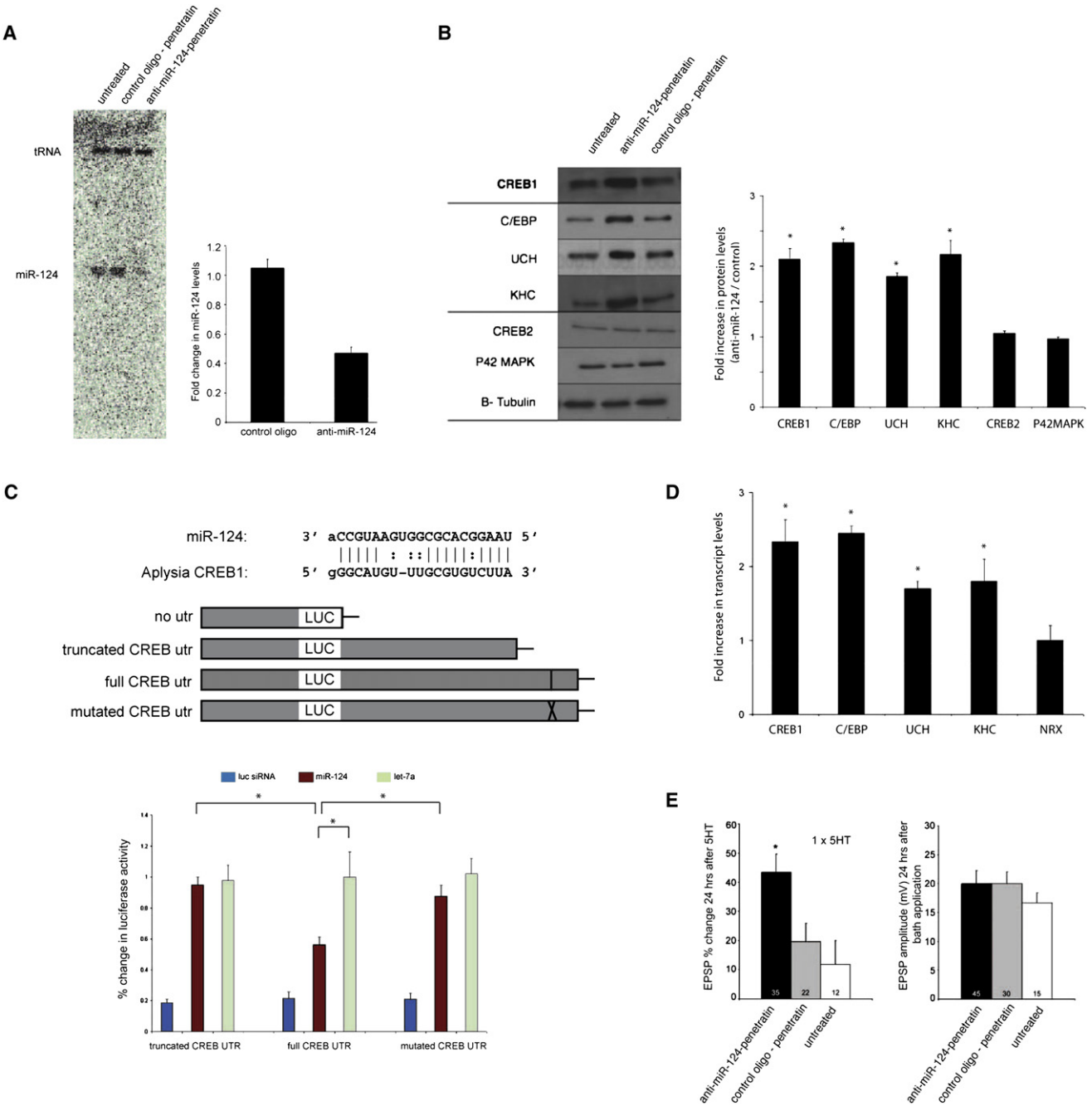


Figure 6. miR-124 Directly Regulates CREB and Facilitates the Switch that Converts Short-Term into Long-Term Synaptic Facilitation

(A) A northern blot with 10 μg total RNA from *Aplysia* pleural ganglia loaded in each lane, after treatment with either 2′-O-methyl oligonucleotides antisense to miR-124 conjugated to penetratin or with 2′-O-methyl control oligonucleotides antisense to miR-194 or with vehicle alone. Blots are probed for miR-124 to show efficient and specific knockdown of miR-124 by penetratin conjugates. Blots were reprobed to detect tRNA, without stripping, to verify equal loading of all lanes. Level of knockdown is quantified by taking the mean percentage reduction of antisense miR-124 as compared to antisense miR-194 over four independent trials ±SD.

(B) A western blot loaded with 15 μg total protein in each lane, after treatment with either 2′-O-methyl oligonucleotides antisense to miR-124 conjugated to penetratin or with 2′-O-methyl control oligonucleotides antisense to miR-194 or with vehicle alone. CREB1 and three of its downstream targets (kinesin heavy chain [KHC], CAAT enhancer binding protein [C/EBP], and ubiquitin C-terminal hydrolase [UCH]) are upregulated after miR-124 inhibition. CREB2 and MAPK are not altered by miR-124. All blots were reprobed with beta tubulin to verify equal loading of all lanes. Changes in protein levels were quantified as a ratio of band intensity between anti-miR-124 and control oligo treatment after each was normalized to the loading control. Data are shown as a mean of ten independent trials for CREB1 and at least five for all others ±SD.

pathways once activated. We also noticed that CREB, in turn, may be able to regulate miR-124 expression levels since there are several putative CREB binding sites in the presumed promoter region upstream of the *Aplysia* mir-124 gene (Figure S2). Although *Aplysia* and mammalian systems have clear differences in the complexities of their CNS, and also even in the types of neurotransmitters used during long-term memory processes, the underlying calcium-induced signaling pathways (including cAMP, PKA, MAPK, and CREB) and their functions are very much shared (reviewed by Kandel, 2001). It is therefore very likely that miR-124 is activity-regulated in the mammalian hippocampus and regulates CREB in much the same way as observed here, especially in light of the fact that the mammalian CREB1 UTR bears a conserved miR-124 target site as predicted by targetscan (Lewis et al., 2003), which was recently confirmed as a site directly bound by Argonaute in mouse brain (Chi et al., 2009).

In summary, we have identified a comprehensive set of brain-enriched miRNAs in *Aplysia*, many of which can be regulated by the neuromodulator serotonin, signifying potential roles in learning-related synaptic plasticity. Specifically, we demonstrated that brain-specific miR-124 responds to serotonin by derepressing CREB and enhancing serotonin-dependent long-term facilitation. This initial study compels the exploration of how neuromodulators act through small RNAs during various forms of plasticity and whether some act locally at synapses. We also have evidence that some 5HT-regulated *Aplysia* miRNAs regulate plasticity-related genes involved in local protein synthesis at the synapse (F.F., P.R., and E.K., unpublished data). The likelihood of a coordinated set of miRNAs combinatorially regulating events at the synapse makes possible a new and rich layer of computational complexity that could be responsible for the emergence of discrete and long-lasting states of activity at the synapse.

EXPERIMENTAL PROCEDURES

Small RNA Cloning, Sequencing, and Annotation

All animals were obtained from the NIH/University of Miami National Resource for *Aplysia*. Prior to dissection, animals were anesthetized by injection of isotonic $MgCl_2$ (337 mM) at a volume of 50%–60% of their body weight. RNA was isolated from dissected tissue according to the standard Trizol (Invitrogen) protocol, with additional extractions with acidic phenol:chloroform: isoamyl alcohol, and finally again with chloroform before precipitation in 3 volumes of ethanol. Starting RNA amounts for each library were as follows: whole animal, 250 μg; CNS, 90 μg; pleural ganglia, 45 μg; abdominal ganglia, 90 μg. Small RNA cloning was performed as described (Hafner et al., 2008). Preadenylated 3′ adapters were used, along with a truncated T4 RNA ligase, Rnl2 (Ho et al., 2004), to avoid circularization of the microRNAs during 3′ adaptor ligation. 5′ adaptor ligation was carried out at standard conditions with T4 RNA ligase (Fermentas Life Sciences) in the presence of ATP. The adaptor sequences were as follows: 3′ adaptor, AppTTTAACCCGGCAC CCTC; 5′ adaptor, ATCGTaggcaccugaaa. After both ligation steps, and following RT-PCR, the markers were removed from the samples by PmeI digest. The samples were again PCR amplified, concatenated, and then cloned into the commercially available TOPO 2.1 vector as described (Hafner et al., 2008). A total of about 250,000 reads each were obtained for the whole animal and CNS libraries by 454 sequencing (454 Life Sciences, Connecticut, USA). Traditional Sanger sequencing was used (Columbia Genome Center, New York, USA) to obtain ~15000, 20000, and 30000 reads each from the abdominal, pleural, and CNS libraries, respectively.

For annotation, various databases were used (referenced in Supplemental Experimental Procedures) to obtain a collection of known annotated RNA sequences. We generated and added to this a set of *Aplysia* tRNAs and rRNAs (explained further in Supplemental Experimental Procedures). All cloned sequences were aligned to this annotation database, and sequences with no annotation but with appropriate length and processing requirements were considered miRNA candidates. These candidates were aligned to *Aplysia* trace archives, around which contigs were assembled, and the resulting miRNA precursors were entered into the annotation database for miRNA annotation. Sequenced clones were again aligned to the database for annotation, and this recursive process continued until all miRNAs were cataloged and manually curated. All procedures pertaining to sequence extraction, annotation, contig assembly, building of miRNA precursors, families, and orthology tables are further discussed in Supplemental Experimental Procedures.

In Situ Hybridization

To prepare cultured neurons for in situ hybridization, cells were fixed in 4% paraformaldehyde in artificial seawater for 15 min at room temperature and then washed with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then endogenous peroxidase activity was quenched using 3% H_2O_2 for 20 min, following which a 10 min acetylation step was performed, all at room temperature, with quick 1× PBS washes between each step. Prehybridization was carried out for 1 hr at 42°C in 50% formamide, 5× SSC, 5× Denhardt's solution, and 0.1 mg/ml each of salmon sperm DNA and yeast tRNA. Hybridization was then carried out for 4 hr, at 42°C, with 60 ng of 3′ end-labeled digoxigenin (DIG) probe per 150 μl of hybridization solution. The first wash was done using 5× SSC for 20 min at 42°C, and two subsequent washes were done with 0.5× SSC for 10 min each at the same temperature. The probes were then blocked for 1 hr in 10% (in TBS) heat-inactivated sheep serum at room temperature, incubated in 1:1000 dilution of anti-DIG-POD antibody (roche) in 1% sheep serum (in TBS) overnight at 4°C, then labeled for detection with TSA-Plus FITC system (PerkinElmer) according to the manufacturer's instructions.

Pharmacological Treatment, Northern and Western Blot Analysis

Whole CNS or pleural ganglia were dissected in ice-cold sea water, desheathed, and kept in L-15 supplemented with glutamine for 24 hr at

(C) The miR-124 target site in the *Aplysia* CREB1 UTR is shown, along with the constructs used for the following reporter assay. A luciferase reporter (100 ng) bearing the CREB UTR (full CREB UTR) is repressed by 45% when cotransfected with miR-124 duplex (5 pmol) in HEK293 cells. The same reporter, when cotransfected with let-7, shows no significant change in expression levels. Luciferase reporters bearing the CREB UTR with a 2 nt mutation in the miR-124 binding site (mutated CREB UTR) and a truncated CREB UTR that is missing the entire miR-124 binding site (truncated CREB UTR) are not significantly affected by cotransfection with miR-124 duplexes. An siRNA directed against the luciferase firefly gene (luc siRNA), a positive control, was able to repress all constructs containing the firefly gene by 80%. Each data point is expressed as a ratio of renilla to firefly activity, normalized to the change in luciferase activity when plasmids are transfected alone without miR duplexes. Data are shown as a mean of eight independent trials ±SD.

(D) Fold increase in transcript levels of CREB1, KHC, UCH, and C/EBP after inhibition of miR-124, as detected by real-time reverse-transcription PCR. Proteins downstream to CREB (KHC, UCH, and C/EBP) have significantly increased transcript levels, whereas a transcript not known to be an immediate-early gene of CREB, neurexin, shows no such increase. Transcript levels were normalized to GAPDH, and data are presented as a mean of five independent trials ±SD.

(E) Bar graph showing the average percentage synaptic facilitation measured at 24 hr after treatment with a single pulse of serotonin in cultures that had been preincubated with either the penetratin-conjugated miR-124 inhibitor or the control miR-194 inhibitor, as well as of untreated controls. The observed differences in the facilitation between the different groups were not due to differences in the basal strength of the synaptic connections as tested before 5HT application. Percent changes in EPSP are shown as an average of many independent trials (the exact numbers for each condition shown within each bar) ±SD.

18°C. Serotonin treatment was performed with five pulses of 10 μM 5HT for 5 min each at 20 min intervals. All drug treatments were done at a concentration of 10 μM in L-15 and bath applied to CNS for 30 min prior to treatment with five pulses of 5HT. The inhibitors used in this study are as follows: KT5720 (PKA inhibitor, Calbiochem), U0126 (MAPK inhibitor, Sigma), MG-132 (Proteasome inhibitor, Sigma), and bisindolylmaleimide (PKC inhibitor, Calbiochem). Inhibition of miRNAs was carried out using penetratin-conjugated 2′-O-methyl antisense oligonucleotides. These oligonucleotides were ordered (Dharmacon, Inc.) with 5′ thiol modification and incubated, with equimolar concentrations of activated penetratin (Qbiogene, Inc., PENA0500) featuring an N-terminal pyridyl disulfide functional group, for 15 min at 65°C, then 1 hr at 37°C. The penetratin-conjugated antisense oligonucleotides were checked for conjugation efficiency by Coomassie staining on 17% polyacrylamide gels, and knockdown efficiency by northern blot. 150 μl of 200 nM penetratin-conjugated oligonucleotides were then applied to desheathed pleural ganglia in Eppendorf tubes, for a minimum of 4 hr before washout, and kept in L-15 with glutamine for a minimum of 24 hr before harvesting RNA or protein.

Northern blot analysis was performed as described (Landgraf et al., 2007). Between 20 and 40 μg of total RNA was loaded per lane, the probes were 5′^{32}P-radiolabeled 21- or 22- nt oligodeoxynucleotides complementary to the predominantly cloned miRNA sequence, and the hybridization was done at 42°C. To monitor equal loading of total RNA, the blots were reprobed with 5′-TGGAGGGGACACCTGGGTTCGA-3′ to detect tRNA.

For western blotting, protein was isolated from desheathed pleural ganglia by incubating and rotating in SDS-urea lysis buffer (50 μl per two pleural ganglia) for 15 min at room temperature followed by centrifugation at 13000 RPM for 10 min, and collection of the supernatant. Protein samples were then quantified using the BCA kit (Pierce Biotechnology) and 15 μg were loaded for western blot analysis. The following commercial antibodies were used: CREB1 (New England Biolabs) 1:1000, MAPK (Cell Signaling Technology) 1:1000, C/EBP (Santa Cruz Biotechnology, Inc.) 1:1000, UCH (Biomolecules) 1:1000, Tubulin (Sigma-Aldrich) 1:10000. KHC and CREB2 were rabbit polyclonal antibodies raised in the laboratory. Following incubation with primary antibodies, a 1:10,000 dilution of either anti-rabbit or anti-mouse antiserum was used to detect protein bands by chemiluminescence (Amersham Biosciences).

Quantitative Real-Time PCR

Ganglia were dissected, maintained, and treated as described above. RNA was isolated according to the traditional Trizol (Invitrogen) method. After the isopropanol precipitation, the pellet was washed with 70% ethanol, and converted into cDNA using random hexamer priming and Superscript III (Invitrogen Life Sciences). Primers were selected using the Primer Express software (Applied Biosystems) and chosen to ensure no significant amplification of DNA. The primer sequences were as follows: CREB1: TCTCGGAAACGG GAATTACG; TTCCCTGGCTGCCTCTCTATT. KHC: GTTCGGCCTCTGAATCA GTCA; TTGAGAACAAACTTGCTGCCA. C/EBP: GCCCCCTACTCCACAAAG TCT; CTGGCCCTCTTATCCACGTACT. UCH: GTACATGCCCTGGCGAACA; CTTTGCAGCATCGAAGGGA. NRX: ACCCTCCAGATCGACGCTG; TGGGCTT GTTTGCCTGTTG. Pre-miR-124: CCCATTTGTGTTCACTGTGTG; ACCGCGT GCCTTAATAGTGT. GAPDH: GCCTACACCGAGGACGATGT; GGCGGTGTCT CCCTTAAAGTC.

Reporter Assays

HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) in 96-well plates (250,000 cells/well) at 50% confluency with 100 ng psicheck-2 dual promoter plasmid (Promega), with renilla bearing the synthetic UTRs, and firefly serving as the internal transfection control. Cells were simultaneously transfected with or without 5 pmol miRNA duplex. Firefly and Renilla luciferase activities were measured 36 hr after transfection with Dual-luciferase assay (Promega).

Cell Culture, Injections, and Electrophysiology

Cell cultures of *Aplysia* neurons were prepared as previously reported (Schacher and Proshansky, 1983; Montarolo et al., 1986). For intracellular injections, miRNA *mimic* and *inhibitors* (Dharmacon, Inc.) were resuspended in nuclease-free water (Ambion, Inc.) to obtain a final concentration of 5.0–5.5 μM, aliquoted and stored at –20°C. Before injection, the solutions were thawed and combined with 10% v/v 2M KCl and 5% v/v of a saturated fast green solution to monitor the intracellular injection under both electrophysiological and visual control. Two to five μl of each solution were loaded into the tip of beveled sharp glass microelectrodes, and after impalement, sensory neurons were injected by seven to ten pressure pulses (1-10 psi; 300-500 ms) delivered through a pneumatic picopump (PV820; WPI). In some experiments, *Aplysia* sensorimotor cocultures were pretreated with penetratin-conjugated inhibitors before electrophysiological recording and 5-HT application. To this aim, the existing culture medium was gently exchanged with a 200 μl solution of a penetratin-conjugated miRNA inhibitor that has been diluted in L-15 to a final concentration of 200 nM. The cells were then maintained for 24 hr at 18°C and subsequently tested electrophysiologically after adding 0.5–1 ml of fresh L-15 into the culture dish.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at http://www.cell.com/neuron/supplemental/S0896-6273(09)00682-5.

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