

# Roles for small noncoding RNAs in silencing of retrotransposons in the mammalian brain

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Piwi-interacting RNAs (piRNAs), long thought to be restricted to germline, have recently been discovered in neurons of *Aplysia*, with a role in the epigenetic regulation of gene expression underlying long-term memory. We here ask whether piwi/piRNAs are also expressed and have functional roles in the mammalian brain. Large-scale RNA sequencing and subsequent analysis of protein expression revealed the presence in brain of several piRNA biogenesis factors including a mouse piwi (*Mili*), as well as small RNAs, albeit at low levels, resembling conserved piRNAs in mouse testes [primarily LINE1 (long interspersed nuclear element1) retrotransposon-derived]. Despite the seeming low expression of these putative piRNAs, single-base pair CpG methylation analyses across the genome of *Mili*/piRNA-deficient (*Mili*<sup>-/-</sup>) mice demonstrate that brain genomic DNA is preferentially hypomethylated within intergenic areas and LINE1 promoter areas of the genome. Furthermore, *Mili* mutant mice exhibit behavioral deficits such as hyperactivity and reduced anxiety. These results suggest that putative piRNAs exist in mammalian brain, and similar to the role of piRNAs in testes, they may be involved in the silencing of retrotransposons, which in brain have critical roles in contributing to genomic heterogeneity underlying adaptation, stress response, and brain pathology. We also describe the presence of another class of small RNAs in the brain, with features of endogenous siRNAs, which may have taken over the role of invertebrate piRNAs in their capacity to target both transposons, as well as protein-coding genes. Thus, RNA interference through gene and retrotransposon silencing previously encountered in *Aplysia* may also have potential roles in the mammalian brain.

piwi-interacting RNA | transposon | DNA methylation | behavior | endogenous siRNA

Piwi-interacting RNAs (piRNAs) are 26- to 32-nt small noncoding RNAs that associate with the piwi clade of the Argonaute family of proteins and whose primary functions in mammals are associated with the silencing of transposons in the germline through de novo DNA methylation (1–4). Transposons constitute 44% of the human genome and could when active contribute to genetic heterogeneity, to alteration of behavior during adaptation, and to responses to stress. Somatic retrotransposition and its associated insertional mutagenesis have particularly important implications for brain disorders and are often associated with schizophrenia, Rett syndrome, and neurodegenerative disorders (5–7). The LINE1 (long interspersed nuclear element1) family of retrotransposons in particular is active in human and mouse hippocampus (8, 9).

Although it has long been thought that piRNA expression and function are restricted to the germline, our laboratory and others have previously found that the piRNA pathway is functional in invertebrate neurons as well (10, 11). *Aplysia* neurons, in particular, have a strikingly abundant expression of piRNAs (contributing to at least 5% of the total noncoding RNA population);

they in turn have unique patterns of biogenesis (with most piRNA clusters containing one dominantly expressed piRNA) and predominant nuclear localization (10). Furthermore, *Aplysia* piRNAs have the function of guiding epigenetic modification to specified gene promoters based on sequence homology. Specifically, one particular piRNA in *Aplysia*, piR-F, contributes to the persistence of long-term learning-related synaptic plasticity by means of epigenetic silencing (DNA methylation) of the *CREB-2* promoter, a major inhibitory transcription factor, that is an inhibitory constraint on learning-related synaptic plasticity. Furthermore, loss of the proteins from the piRNA pathway in the mushroom bodies of *Drosophila*, which are involved in learning and memory, lead to specific disruption of memory-related genes via enhanced transposition into memory-related gene loci (11). Based on these convergent findings on the roles of piRNAs in the regulation of neuronal function in invertebrates, we wondered whether piwi proteins and piRNAs might also be expressed and have function in the mammalian brain.

We also wanted to explore whether neuronal endogenous siRNAs (endo-siRNAs) targeting protein-coding genes known to be involved in odor adaptation in *Caenorhabditis elegans* and olfactory discrimination in mouse (12, 13) and endo-siRNA-mediated silencing of LINE1 demonstrated in human cultured

## Significance

Piwi-interacting RNAs (piRNAs), a class of small noncoding RNAs whose primary function in mammals is silencing germline-resident retrotransposons, are also expressed in the *Aplysia* brain, where they play roles in memory storage. Transposons constitute a bulk of the genome and, when activated, can cause genomic instability and variability by integrating into various gene loci, including those in the mammalian brain, where they can modulate stress response and adaptive behavior and contribute to brain pathology. We show that small RNAs with conserved piRNA-like features are present in the mouse brain. In addition, we describe another class of small noncoding RNAs with features of endogenous siRNAs, which along with putative piRNAs could play critical roles by silencing brain-expressed retrotransposons contributing to mammalian behavior.

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cells (14) are present in the mammalian brain. Endo-siRNAs are a 20- to 24-nt class of noncoding RNAs that are either derived from gene/pseudogenes or retrotransposons using the canonical RNA interference machinery and thought to be generated either from bidirectional transcription units or inverted repeats in ES cells as well as in the female germline (15–17).

Based on large-scale sequencing of noncoding RNAs in mice, we find that both small RNAs with piRNA- and endo-siRNA-like features are expressed in the mammalian brain. Sequencing of coding regions, as well as determination of protein expression, revealed that Mili, one of the three mouse piwis, is expressed in the brain at low level. Using Mili-deficient (*Mili*<sup>-/-</sup>) mice, we further identify distinct LINE-1 promoter regions that are preferentially hypomethylated in *Mili*<sup>-/-</sup> brain tissues. Behavioral analyses using *Mili* mutant mice reveal that the piRNA pathway may have roles in locomotory and exploratory drives and normal anxiety-like behavior. Thus, our results suggest that small noncoding RNAs may have previously unexplored epigenetic roles in the mammalian brain.

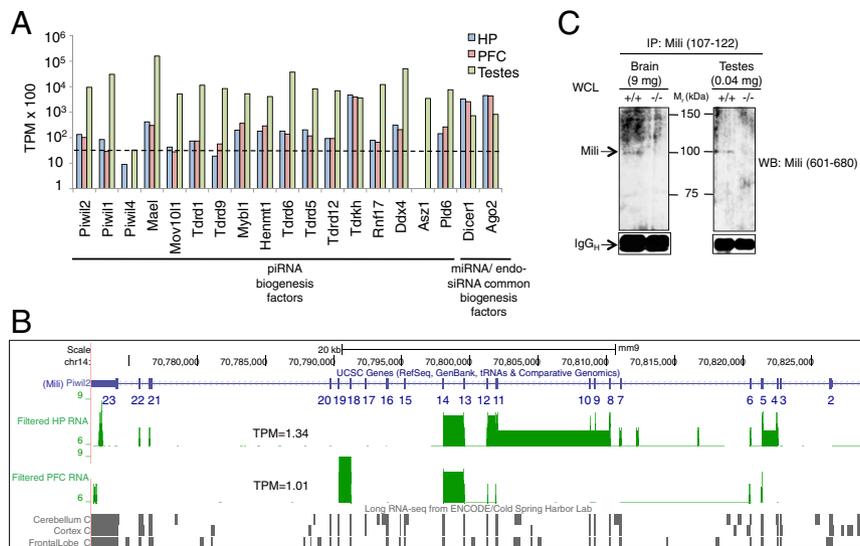
## Results

**piRNA Biogenesis Machinery Including Mili (Piwi) Is Expressed in the Mouse Brain.** We first assessed whether components of the piRNA biogenesis machinery are present in the mouse brain. In mice, there are three piwi genes, *Mili* (*Piwil2*), *Miwi* (*Piwil1*), and *Miwi2* (*Piwil4*), which exhibit distinct but overlapping expression patterns during testes development (3, 18, 19). Although *Miwi2* is expressed from embryonic day 15.5 (E15.5) to the early postnatal period, *Miwi* is only expressed during the pachytene stage after postnatal day 14, and this expression persists in adult testes (20). On the other hand, *Mili* is expressed throughout, from germ cells at E12.5 through postnatal to the adult stages of testes development (1, 21, 22). The fact that *Mili* is associated with all three developmental stage-dependent pools of piRNAs, prenatal, prepachytene, and pachytene piRNAs, suggests that *Mili* plays a central role in the piRNA pathway. Appropriately, testes of *Mili*<sup>-/-</sup> mice lack any detectable piRNAs and possess

hypomethylated retrotransposons, leading to their derepression (1, 2, 4, 18, 22).

RNA-seq analyses of poly(A)-selected cDNA libraries from total RNA isolated from hippocampus and prefrontal cortex (PFC) revealed the presence of many of the proteins involved in piRNA biogenesis, including endoribonucleases such as maelstrom (*Mael*) (23) and phospholipase D6 (*Pld6*) (24), methyltransferase responsible for adding a 2'-O-methyl group at the 3' end of piRNAs, the HEN methyltransferase 1 (*Henmt1*) (25), RNA helicase such as *Ddx4* (26), and Mili-interacting Tudor domain-containing protein (*Tdrd*) 1 and 5 involved in repression of LINE1 retrotransposons (27, 28) (Fig. 1A). Of the three piwis, the *Mili* (*Piwil2*) transcript was detected in tissues from hippocampus and PFC (Fig. 1A), although at two orders of magnitude lower than levels found in testes, which was further supported by our analyses of three deposited brain libraries in the encyclopedia of DNA elements (ENCODE) (Fig. 1B and Fig. S1). Although these expression levels were considerably lower than that found in *Aplysia* sensory neurons, this difference could be attributed to the complexity and heterogeneity of cell types in the mammalian brain and the proportion of cells in the brain that may express these piRNA biogenesis factors. Among the known piRNA pathway factors, expression of *Miwi2* (*Piwil4*) and *Asz1* remain undetected in the brain tissues (Fig. 1A and Dataset S1).

To determine whether *Mili* was expressed at the protein level, we performed Western blotting from whole brain lysates and from cytosolic fractions; both failed to reveal full-length *Mili* protein. Instead, we could detect a putative N-terminally truncated form of *Mili*, piwil2-like protein-60 (PL2L60) at a significant level in WT brain cytosol, but depleted (although not completely absent) from *Mili*<sup>-/-</sup> brain cytosol (29) (Fig. S2). The PL2L60 protein is believed to have an incomplete PAZ domain but intact MID and PIWI domains and could still retain the capacity of RNA binding, target recognition, and suppression (30, 31). Immunoprecipitation followed by Western blotting from whole brain lysates further revealed that full-length *Mili* protein was present in the brain at a low level, approximately two



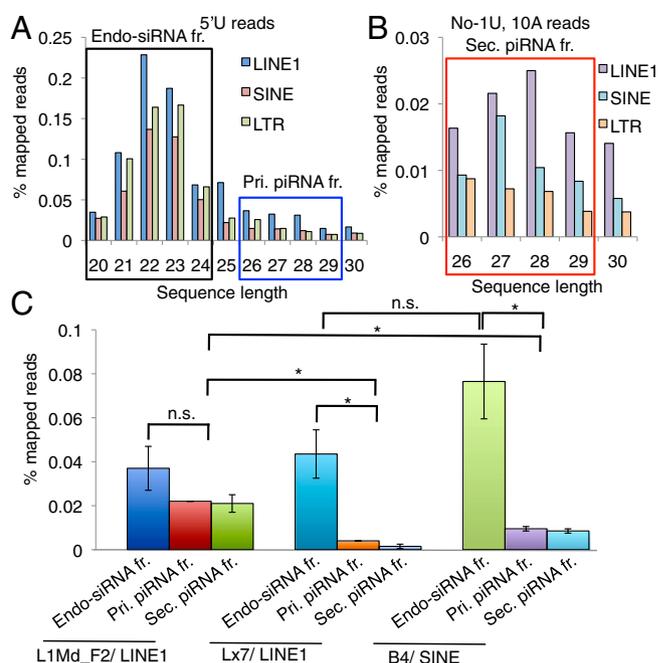
**Fig. 1.** Expression of the piRNA biogenesis machinery in the brain. (A) Transcript expression analyses by RNA-Seq. The dotted line represents baseline of expression based on TPM score for *Piwil4*, a transcript absent in adult testes (3). (B) Visualization of *piwil2* (*Mili*) transcript alignment from poly(A) selected (green) and from ENCODE long RNA-seq contigs (gray). Corresponding exons of the *Mili* gene were identified by numerical values from 1 to 23. Note, number of reads in exon 23 (noncoding) accounts for 61–63% of all reads mapping to the *Mili* transcript in all tissues. (C) *Mili* protein expression. Whole cell lysates from brain or testes, from either WT (+/+) or *Mili*<sup>-/-</sup> (-/-) mice were immunoprecipitated using the N-terminal anti-*Mili* antibody and Western blotted using the C-terminal anti-*Mili* antibody. The experiment was carried out two to three times. Note, brain lysates were used at 225-fold higher than testes for immunoprecipitation.

orders of magnitude lower than its expression in adult testes (Fig. 1C).

We, however, failed to detect evidence for PL2L60 expression from RNA-Seq analyses. We calculated transcripts per million (TPM) for coding exons, i.e., exons 1–10 and 11–22, of the *Mili* transcript. The exons 1–10 were not significantly depleted compared with exons 11–22, which would be consistent with an additional 5' truncated transcript corresponding to an N-terminally truncated isoform. The TPM ratio (exon 1–10/exon 11–22) was 1.3 in hippocampus, 2.9 in PFC, and 0.9 in testes. The signal observed in 3' regions of the PFC sample in the visual alignment is largely due to contaminating intronic reads (Fig. 1B). It is possible, however, that presence of several other putative serially N-terminally truncated *Mili* isoforms (and their serially 5' truncated transcripts), as postulated (29), may have complicated interpretation of our RNA-Seq data. Although we failed to detect *Miw2* expression by RNA-seq analyses, others have reported expression of its human homolog (*piwil4*) protein in brain tissues (32). We suspect that our failure to detect *Miw2* transcript in our RNA-Seq analyses could either be due to the instability of *Miw2* mRNA or to the lack of sequencing depth. It is also possible that compared with *Mili*, *Miw2* was expressed in fewer cell types and also in a temporally restricted manner. Nevertheless, because *Mili* was shown to act upstream of *Miw2*, and is also capable of functioning independently of *Miw2*, our subsequent studies using *Mili*<sup>-/-</sup> mice should provide a comprehensive functional readout of the contributions of the piRNA pathway in mammalian brain (1, 33).

**Retrotransposon-Derived Small RNAs with piRNA-Like Features Are Present in Mammalian Neurons.** We next investigated whether piRNAs could be present in hippocampal neurons. We profiled small RNAs from primary neurons by preparing two small RNA cDNA libraries from total RNA followed by deep sequencing and bioinformatic analyses. Although we size-selected the libraries by gel extraction of small RNAs over 24 nt, we were only able to minimize but not completely exclude miRNAs from cloning. We thus analyzed the entire length of the small RNA fraction (18–35 nt) (Fig. S3A and B and Dataset S2). Compared with the entire small RNA fraction, the 26- to 32-nt fraction was particularly enriched for structural [rRNA, tRNA, snRNA, and small nucleolar RNA (snoRNA)] (55.8%) and repeat-derived small RNAs (30.3%), although gene-derived small RNAs were also present (6.7%) (Fig. S3B). Further characterization of potential piRNAs by mapping non-miRNA, nonstructural RNA, and non-repeat-derived reads to National Center for Biotechnology Information (NCBI) Refseq and by requiring size specification (26–32 nt) together with a starting uridine base (5'U), failed to find evidence for gene-derived piRNAs (1, 32) (Fig. S4). We also failed to find evidence for uniquely mapping, intergenic cluster-derived pachytene piRNAs in brain (19) (Fig. S5). However, applying the same criteria for defining piRNAs by mapping of non-miRNA, nonstructural RNA to the repeatmasker database revealed an abundance of retrotransposon-derived piRNA-like small RNAs (Fig. S4).

Further analyses of repeat-derived read sequences revealed a depletion of short interspersed nuclear element (SINE) (1.9%) and long terminal repeat (LTR) (1.4%), but not LINE (5.3%) retrotransposon-derived clones from the 26- to 32-nt fraction compared with the entire fraction (Fig. S3C). Of the LINE1-derived piRNA-like small RNAs, the highest abundant reads were produced from L1Md\_F2 (Fig. 2A and Dataset S3). Consistent with the “ping-pong” feature associated with biogenesis of LINE1-derived germline piRNAs, a small but distinct peak ~28 nt was visible for neuronal LINE1 clones that lacked 5' uridine but had adenine at the 10th position (No-1U, 10A, or secondary piRNA fraction), again with maximum abundance of L1Md\_F2 (1, 34) (Fig. 2B and Dataset S3). SINE clones also displayed a small peak ~27 nt in the secondary piRNA fraction, with greater



**Fig. 2.** Characteristics of the neuronal piRNA fraction. (A) Size distribution of retrotransposon-derived small RNA clones with 5'U. Primary piRNA fraction was defined as 26- to 29-nt, 5'U clones mapped to retrotransposons. Endo-siRNA fraction was defined as 20- to 24-nt, 5'U clones (on removal of miRNAs and structural RNAs) mapped to retrotransposons and genes/pseudogenes (Fig. S4). (B) Secondary piRNA fraction was defined as 26- to 29-nt clones without 5'U but with an adenine (A) at the 10th position. (C) Relative abundance of top subfamilies of retrotransposon-derived small RNAs within various small RNA fractions from two biological replicates. Average  $\pm$  range. Unpaired *t* test was used to test significance of difference between two sets. \**P* < 0.05; n.s., not significant.

abundance of Alu/B2/B4 families suggesting these could have also undergone “ping-pong” cycle something not unambiguously described for these class of piRNAs (1, 4) (Fig. 2B and Dataset S3). Furthermore, consistent with the ping-pong feature, LINE1 and SINE secondary piRNA fractions had bias for small RNAs in antisense orientation (antisense/sense = 1.9 for LINE1, 1.7 for SINE, and 1.0 for LTR), in contrast to the endo-siRNA fraction (antisense/sense = 0.9 for LINE1, 0.6 for SINE, and 0.8 for LTR) (1). These results were consistent with plausible Mili-directed piRNA amplification in the brain similar to what has been described for the fetal germline (1, 34).

In addition to the small RNAs with conserved features of testes piRNAs, we discovered another larger fraction of small noncoding RNAs in brain, the endo-siRNAs sized 20–24 nt (Fig. 2A and Fig. S4). Its increased abundance compared with the piRNA fraction is consistent with a higher expression of endo-siRNA biogenesis factors in brain tissues (Fig. 1A and Dataset S1). We find the endo-siRNAs to be both gene-derived, as well as transposon-derived, and unlike the neuronal piRNA fraction that had a clear bias for LINE1 small RNAs, the endo-siRNA fraction showed equal distribution of all three classes (Fig. 2A and Dataset S3). Furthermore, the L1Md\_F2 subfamily, the most abundant in the piRNA fraction, also constituted the second most abundant LINE1 endo-siRNA subfamily (endo-siRNA fraction:piRNA fraction = 2:1; Fig. 2C and Dataset S3). Interestingly, Lx7, the most-abundant LINE1 subfamily of endo-siRNA, was depleted from the piRNA fraction (endo-siRNA fraction:piRNA fraction = 10:1) (33) (Fig. 2C and Dataset S3). In the same vein, the top expressing endo-siRNA subfamily, B4 SINE, was present at a low level within the piRNA fraction

(endo-siRNA fraction:piRNA fraction = 6:1) (Fig. 2C and Dataset S3), reflecting our initial observation of an overall depletion of SINE class of small RNAs in the 26- to 32-nt fraction (Fig. S3C). These cumulative observations suggested that neuronal piRNA fraction appeared to contribute to a limited set of LINE1 and to a lesser extent, SINE-derived noncoding RNAs in the mammalian brain.

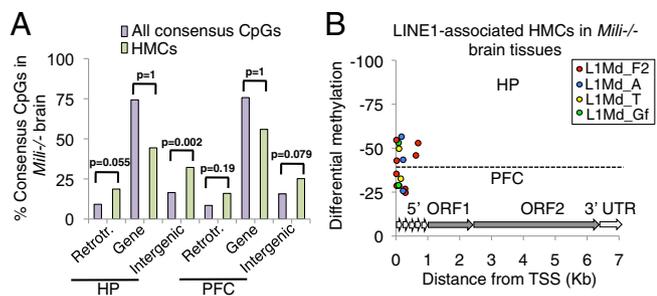
**Mili<sup>-/-</sup> Mice Exhibit LINE1 Promoter Hypomethylation in Their Brain Tissues.** Because piwi/piRNAs promote CpG methylation of retrotransposons, in particular LINE/LTR classes in the mouse germline (1, 2, 4) and the CREB2 promoter in *Aplysia* neurons (10), we investigated the consequences of the loss of the piRNA pathway on DNA methylation in the hippocampus and PFC. We interrogated DNA methylation status of CpGs at a base pair resolution across the genome using enhanced reduced representation bisulfite sequencing (ERRBS) (35).

Consistent with a signature for piRNA pathway activity, we observed an overall DNA hypomethylation in the *Mili<sup>-/-</sup>* brain tissues, with 61.7% differentially methylated cytosines (DMCs) in hippocampi and 63.3% in PFC (Fig. S6A). We thus identified a total of 150 and 169 sex-consensus hypomethylated CpGs (HMCs), respectively, in the mutant hippocampi and PFC. Ninety-eight of these HMCs were also common between mutant tissues. Moreover, 23.3% and 18.9% of these HMCs, respectively, in mutant hippocampi and PFC, also coincided with repeat elements, primarily retrotransposons (Fig. S6B). The majority of HMCs (44.4% in mutant hippocampi and 55.9% in mutant PFC) coincided with normally CpG-rich areas including the protein-coding genes (promoters, introns, and exons), which was likely due to the bias introduced by ERRBS (Fig. S6B). We observed enrichment for HMCs in the intergenic regions (permutation test,  $P = 0.002$ ) and retrotransposons to some extent (permutation test,  $P = 0.055$ ) compared with all consensus CpGs within respective annotation (Fig. 3A). This enrichment was more strongly observed in mutant hippocampi compared with mutant PFC (Fig. 3A). Furthermore, strong hypomethylation was observed in two genomic locations [chromosome 9: 123924637–123925547; chromosome 17: 39011428–39011506; mm10/University of California, Santa Cruz (UCSC)], which accounted for 60% of all intergenic HMCs in the mutant hippocampi (highlighted in Dataset S4). Interestingly, consistent with Mili regulation of LINE1 promoter methylation in the germline (33, 36), all LINE1-associated HMCs in the mutant hippocampi coincided with

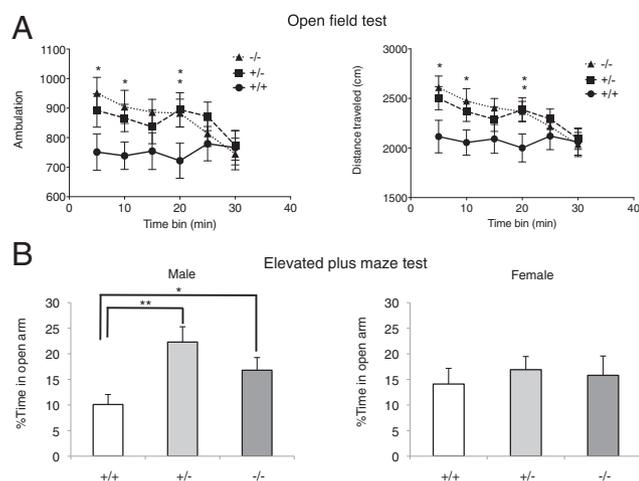
LINE1 promoters (50% of all LINE1 belonged to L1Md\_F2, compared with a baseline representation of 26% in hippocampus) and that mutant hippocampi compared with mutant PFC exhibited a greater degree of LINE1 promoter hypomethylation (Fig. 3B and Dataset S5). These results were consistent with an enrichment of L1Md\_F2-derived piRNA-like small RNAs in the hippocampal neurons (Fig. 2C and Dataset S3). Interestingly, full-length LINE1-promoter associated HMCs were conspicuously present in the *Mili<sup>-/-</sup>* genome with an absence of hypermethylated CpGs in those regions, suggesting Mili-directed piRNA targeting of these LINE1 promoters (Fig. S7). Indeed, DMCs in WT PFC exhibited a greater degree of variation in methylation and a stronger sex bias compared with those in WT hippocampus (Fig. S7). In contrast, hippocampal LTR-associated HMCs were fewer and more evenly distributed throughout the length of the transposon as described for the germline (Dataset S5) (33, 36), consistent with the paucity of LTR-derived sequences in neuronal piRNA fraction (Fig. 2A and B and Dataset S3). Although enrichment of HMCs for genes was not significantly different from baseline CpGs based on permutation test (Fig. 3A) and that there was also no evidence for gene-derived piRNAs in our small RNA Seq datasets (Fig. S4), we nevertheless documented HMCs associated with six genic promoter regions in *Mili<sup>-/-</sup>* mice (Dataset S6). Three of those six genes were involved in the stress response (Dataset S6). Understanding the role of intergenic CpG methylation is of great biological interest and the existence of genome wide changes in retrotransposon regions caused by the Mili-directed piRNA pathway in adult brain (hippocampus and PFC) suggests that neuronal piRNAs may have potential regulatory roles in gene expression in hippocampus related to behavioral performance, such as navigation, memory, and anxiety.

**Mili<sup>-/-</sup> Mice Display Hyperactivity and a Reduced Anxiety-Like Behavior.**

As a first step in determining whether the Mili-directed piRNA pathway in adult mice has a role in behavior and cognition, we used an open-field test to assess rodent locomotory/exploratory behavior by exposing the animal to a novel environment (a Plexiglas chamber) (37). We first observed that compared with WT (+/+) mice, the *Mili* homozygous (-/-) mutant, and to a lesser extent, heterozygous (+/-) mutant mice displayed enhanced ambulation (Fig. 4A). Moreover, compared with WT mice, *Mili<sup>-/-</sup>* and *Mili<sup>+/-</sup>* mice traveled greater distance overall (Fig. 4A). These results suggested that, in addition to displaying an enhanced locomotory activity, *Mili* mutant mice also showed an enhanced exploratory drive; this hyperactive behavior is sensitive to the number of functional copies of the *Mili* gene (Fig. 4A). Using the elevated plus maze test that measures anxiety in rodents (38), we further observed that *Mili<sup>+/-</sup>* and *Mili<sup>-/-</sup>* males spent more time in the open arm of the maze that is normally avoided by WT males (Fig. 4B). This phenotype was absent, however, in mutant females (Fig. 4B). These results indicate that, in addition to being hyperactive (defined as exhibiting an enhanced locomotory/exploratory drive), *Mili* mutant mice also exhibited a reduced anxiety-like behavior. We, however, could not rule out the possibility that an increased amount of time spent by the mutants in the open arm of the elevated plus maze could in part be contributed by the hyperactive behavior of these mutants. *Mili<sup>-/-</sup>* males exhibited smaller testes and were sterile due to spermatogenic arrest (22). The piRNA pathway specifically targets spermatogenic cells, leading to their paucity, but largely sparing other testicular cell types including Leydig cells, which produce testosterone that has a role in reduction of anxiety. It is interesting that hyperactive behavior was observed in mutant females and that *Mili<sup>+/-</sup>* males with a normal testicular development and function exhibited hyperactive behavior in the open field test and a reduced anxiety in the elevated plus maze test. We thus rationalize that the behavioral phenotype was not secondary to abnormal germ cell development and function in *Mili<sup>-/-</sup>* mice. We, however, cannot rule out the



**Fig. 3.** Characteristics of HMCs in *Mili<sup>-/-</sup>* brain. (A) Enrichment analyses of HMCs within various annotation categories. The relative abundance of each annotation category within HMCs was compared with that over all consensus CpGs in *Mili<sup>-/-</sup>* brain tissues. Statistical significance was determined by Permutation test,  $n = 1,000$  (refer to SI Materials and Methods for detail). (B) Distribution of HMCs along copies of LINE1 in *Mili<sup>-/-</sup>* brain tissues. Each circle represents individual HMC plotted over its distance from LINE1 transcription start site (TSS) vs. differential methylation.  $n = 3$  per tissue per genotype. The dotted line separated HP from PFC, showing greater hypomethylation in mutant HP. Retrovir., retrotransposon.



**Fig. 4.** Effects of the piRNA pathway in behavior. (A) *Mili* mutants exhibited hyperactivity. Locomotory activity and exploratory behavior were, respectively, assessed by monitoring number of beam-breaks inside the chamber (ambulation) and by total distance traveled. (B) *Mili* mutant males displayed a reduced anxiety-like behavior. Time spent in the open arm of an elevated maze for 5 min was assessed separately for male and female mice. Average  $\pm$  SEM; unpaired two-tailed *t* tests; \**P* < 0.05 and \*\**P* < 0.01. *n*  $\geq$  9 per group.

possibility of a potential contribution from epigenetic changes carried over from gametogenesis as opposed to an adult role for piRNAs in the brain.

We could not assess the role for *Mili* and the piRNA pathway in other cognitive processes including learning and memory, because the *Mili* mutants' hyperactivity and reduced anxiety-like behavior would potentially interfere with the mutant's performance in behavioral tasks relevant to learning and memory. Future work using conditional knockout mice, restricted to specific spatiotemporal patterns, may reveal cognitive and other impairments in mice lacking *Mili* and piRNA functionality and will further address direct roles for piRNA pathway in the brain. Because *Mili* has previously been described as a germline-specific gene (32), our results suggest a role for *Mili* in the brain.

## Discussion

**An Evolutionary Constraint in the Choice of Specific Noncoding RNA-Directed Gene Silencing Mechanism Across Species.** In view of our finding of piRNAs in the brain of *Aplysia*, we have begun to explore their possible presence and roles in the mammalian brain. Consistent with our previous report in *Aplysia* (10), we demonstrate in the mouse brain, albeit at a low level, the existence of many components of the piRNA pathway by RNA-Seq, immunoprecipitation, and Western blotting (Fig. 1 and Figs. S1 and S2). Putative piRNA level in the brain was assessed by small RNA-Seq and mirrored low expression levels of piRNA biogenesis machinery in the brain (Fig. 2). We further find that, despite a low level of expression, the putative piRNAs appear to give rise to significant changes in both genome-wide DNA methylation and animal behavior (Figs. 3 and 4).

Although there seems to be specific gene silencing mechanism in the brains of *Aplysia* and mice, there are both qualitative and quantitative differences between the piRNAs found in these two organisms, separated by millions of years of evolution. Qualitatively, *Aplysia* neuronal piRNAs are generated from large intergenic, syntenic clusters, map uniquely to the genome, and can target protein-coding gene (10). In contrast, the putative piRNAs in the hippocampus lack these features and seem instead to be generated from retrotransposon transcripts (Fig. 2 and Dataset S3). In fact, the presence of sense and antisense

transcripts are sufficient (without contributions from large genomic piRNA clusters) for piRNA-directed DNA methylation and gene silencing in the male germline (39). Quantitatively, the putative hippocampal neuronal piRNAs constitute  $\sim$ 0.1% of total noncoding RNAs, in contrast to *Aplysia* neuronal piRNAs, which constitute  $\sim$ 5% of total noncoding RNAs. This apparent difference could be due to several reasons: first, the heterogeneity of cell types associated with the mouse brain compared with the sensory neurons of *Aplysia*; second, the expression in mammalian brain could be transient, activity dependent, and temporally restricted (indeed *Aplysia* piRNAs were severalfold induced by neuromodulatory activity); third, the degradation products of other coding and noncoding RNA may prevent efficient cloning and detection of piRNAs (Fig. S3B), especially because of the bias introduced in traditional cloning methods in failing to incorporate piRNAs that are 2'-O-methylated at their 3' ends (this can be overcome in the future with periodate oxidation/ $\beta$ -elimination step (10), along with Mili-immunoprecipitation (1), to enrich for piRNAs before cloning). Finally, and perhaps most importantly, other noncoding RNAs such as gene/pseudogene-derived endo-siRNAs have likely taken up the role of invertebrate piRNAs (Fig. S4). A substantial portion of mouse brain endo-siRNAs are nontransposon, or repeat derived and potentially target genes for epigenetic silencing (Fig. S4). The significant expansion of the mammalian miRNA pool, as well as the ability of a single miRNA to target multiple genes, may fulfill the range of gene targeting accomplished by invertebrates through diverse noncoding RNA species (40).

Importantly, piRNA-directed silencing of retrotransposons (rather than individual protein-coding genes) may have a selective advantage in higher organisms. For example, piRNA-directed transposition in *Drosophila* neurons seems to affect genes that are dedicated to a similar cognitive process such as encoding memory (11). Future studies directed to identify retrotransposed genes in *Mili*<sup>-/-</sup> mice should help to elucidate important roles for piRNAs in plasticity and behavior.

**Brain-Expressed Retrotransposon Transcripts Are the Likely Source of piRNA Production.** In the mouse germline, primary piRNAs are mostly in sense orientation with respect to transposon mRNAs and derived from these transcripts instead of from piRNA clusters (1). These primary piRNAs subsequently recognize antisense transposons to further generate secondary piRNAs in antisense orientation only to be used as substrates for generating more primary piRNAs, in a typical ping-pong amplification loop that absolutely requires *Mili* (1). Because the initiation of the ping-pong cycle relies on the expressed transcripts rather than the piRNA clusters, this might be the case in the brain. Although we did not find evidence for *Miwi2* expression in the brain (despite its expression in human brain and in other tissues), we speculate that *Mili* has taken over the role of *Miwi2* in its capacity to silence retrotransposons. Indeed, *Miwi2* catalytic activity (but not *Mili* catalytic activity) is dispensable for production of secondary piRNAs and that cycles of intra-*Mili* secondary piRNA production cause piRNA amplification required for LINE1 silencing (34).

Earlier studies found that LINE1 can actively retrotranspose in *Drosophila*, mouse, and human brain (9, 11, 41). The LINE1 promoter is active in neural precursor cells differentiating into neurons (9, 42). Thus, the hippocampus, one of the two regions of the adult brain where new neurons are generated, may not be the only area with a detectable piRNA expression. The link between neurogenesis and LINE1 activation can be further reinforced by the fact that human hippocampal neurons have an estimated 13.7 somatic LINE1 insertion per neuron (43), compared with <0.6 unique insertions per neuron from cortex and caudate (44). Consistent with the notion that piRNAs might function in a mitotically active cellular niche, the mRNA for

PL2L60 (accession no. AK027497) was indeed identified in human neuronal precursor cells, and both Mili and PL2L60 proteins were found to be expressed in the stem cell compartment of testes and in cancer stem cells (45, 46). Finally, transposition has both beneficial and deleterious effects on human health (47). As opposed to its proposed role in homeostatic mechanism to environmental stress and adaptive behavior (48), increased LINE1 copy number has been found to be associated with Rett syndrome and schizophrenia (5, 49). Furthermore, depletion of the stress-granule component, transposon-binding protein, TAR DNA-binding protein 43, leads to neurodegeneration in the familial form of amyotrophic lateral sclerosis and frontotemporal dementia (7). Our results thus should provide a mechanism of control of active retrotransposons in the adult brain that has important implications from the generation of genomic diversity in neurons, to stress response and synaptic plasticity, to neurodegenerative and psychiatric illnesses.

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## Materials and Methods

Details on mice and procedures regarding small RNA, poly(A), and gDNA library preparation, sequencing and bioinformatics, hippocampal neuronal culture, RNA isolation, immunoprecipitation, Western blotting, and behavioral analyses are detailed in *SI Materials and Methods*. Animal experiments were carried out following guidelines of the Institutional Animal Care and Use Committee of Columbia University.

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