Review

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New mechanisms in memory storage: piRNAs and epigenetics

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In recent years, a greater understanding has emerged of the role epigenetic mechanisms play in the brain, not only during development, but also in mature neurons involved in long-term memory. The identification of spatially and temporally tuned epigenetic modification of genetic loci during memory storage has revealed the remarkably input-responsive, target-specific, and long-term nature of epigenetic regulation, but the underlying mechanisms have remained elusive. New insight into these mechanisms has come from the study of small RNAs, which have emerged as regulators that can confer sequence specificity to DNA- and chromatin-modifying processes. We discuss advances in the elucidation of the epigenetic mechanisms involved in long-term memory, focusing on the role of small RNAs, and in particular piwi-interacting RNAs (piRNAs), in the epigenetic regulation underlying memory storage.

Memory storage: translating transient signals into lasting information

Synaptic plasticity, the ability to modify the communication between neurons, lies at the heart of our ability to acquire, store, and recall information. Indeed, complex cellular signaling cascades have evolved within neurons in part to preserve a biological history capable of informing future responses. Elucidation of the molecular bases of memory has focused on switches that might transduce transient signals into a more enduring record. Protein-based switches were the first identified, but soon the versatility of RNA, and the stability of DNA, suggested the possibility that both RNAs and epigenetic mechanisms – modifications to chromatin structure that affect gene expression without altering the DNA sequence – contribute to the storage of cellular memory. In particular, recent discoveries regarding the interplay between small RNA-based specificity and epigenetic durability have opened a new avenue for investigation of long-term memory.

The retention of cellular signaling events through changes in the epigenome appears to be an ancient evolutionary motif. The slime mold dictyostellid sits on the cusp of multicellularity and provides a particularly vivid snapshot of this motif: most of its life cycle is spent in a solitary, amoeboid trophic phase, but during starvation, individual dictyostellids secrete cyclic adenosine monophosphate (cAMP), stream together along the chemical gradient [1], and differentiate to form a motile slug. Although cAMP appears only transiently, epigenetic activity maintains long-term differentiation [2]. The epigenetic mechanisms active in dictyostellids, including histone modification, DNA methylation, and small RNA pathways, reappear in neuronal communication and information storage.

In this review, we describe new insights into the role of epigenetic mechanisms in long-term memory, focusing on DNA methylation because of its particular capacity for high target-specificity, dynamic responsiveness to input, and ability to transduce transient stimuli into long-term changes in gene expression. We then discuss the recent and growing body of work on small noncoding RNAs, particularly piRNAs, which are well suited for conferring specificity and responsiveness, and their role in regulating several memory processes. These findings illustrate the importance of small noncoding RNAs as part of the epigenetic control of long-term memory storage.

Epigenetic mechanisms in memory storage

The term 'epigenetics' broadly includes nonheritable but potentially stable changes to chromatin structure. Although epigenetic mechanisms have played an important role in transducing transient environmental cues into long-lasting cellular behavior at least since the evolution of multicellularity, dynamic epigenetic regulation in animals was long thought to be restricted to development and differentiation. However, the discovery that mature neurons use conserved mechanisms for the same purpose of maintaining long-term cellular behavior initiated a new field of research. The markers that encode epigenetic information fall into two broad categories: covalent modifications to histone proteins and modifications to the DNA molecule itself, of which DNA methylation is the most well characterized mode. Both categories have consistently been found to have important roles in memory storage.

Histone modifications in memory storage

Histone proteins compact DNA into nucleosomes rendering it inaccessible to RNA polymerase, and thereby silenced.

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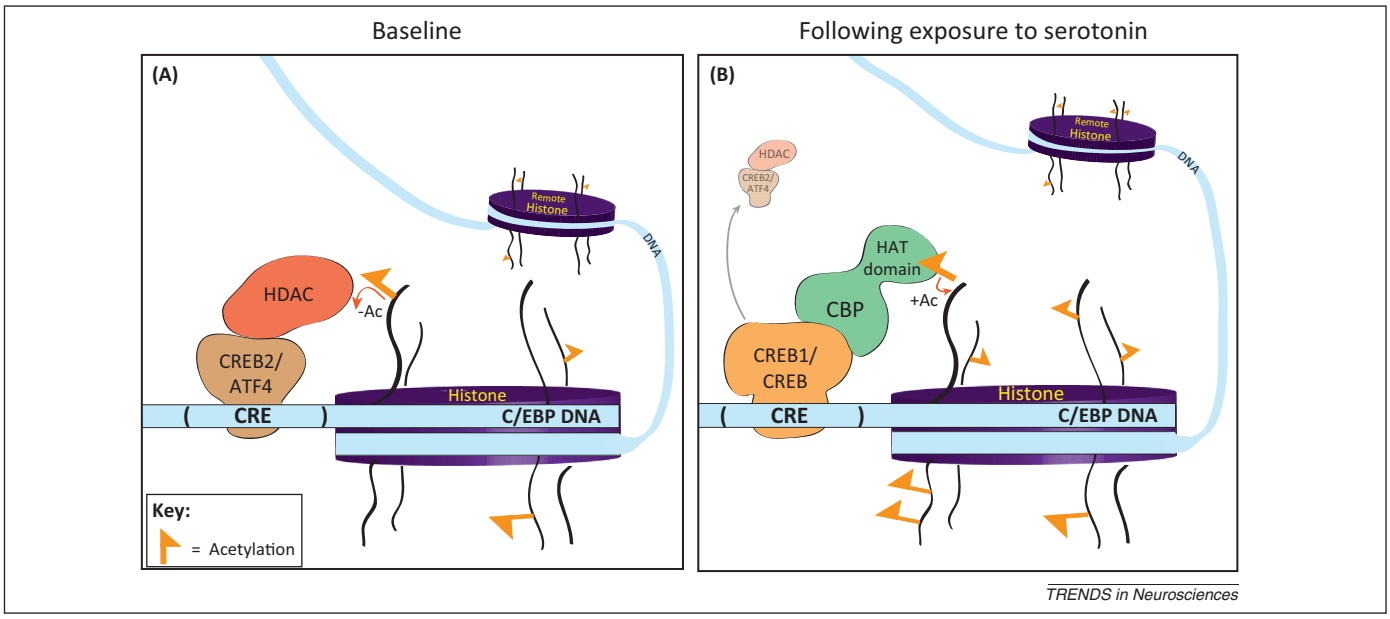

Figure 1. Input responsiveness of chromatin remodeling to regulate expression of *C/EBP*, an immediate early gene (IEG) [5]. **(A)** Represents a neuron at baseline activity. In this state, CREB2/ATF4 binds the CRE element and complexes with HDAC to remove acetyl histone modifications to the histone protein. Deacetylation causes compaction of chromatin and thus inhibition of transcription of IEGs (e.g., *C/EBP*). **(B)** Represents a neuron following exposure to serotonin. In response, CREB1/CREB becomes activated. It competitively binds the CRE and can displace CREB2/ATF4, which is simultaneously inhibited. CREB1/CREB complexes with CBP, whose HAT domain restores acetylation marks. Acetylation causes expansion of the chromatin, and promotes transcription of IEGs. Note: the compaction of chromatin at the IEG may be affected by modifications at a remote histone. Abbreviations: C/EBP, CCAAT/enhancer binding protein; CREB-1,2, cAMP response element-binding protein-1,2; ATF4, activating transcription factor 4; CRE, cAMP response element; HDAC, histone deacetylase; CBP, CREB-binding protein; HAT, histone acetyltransferase; AC, acetylation.

Histones are subject to an array of post-translational modifications of specific residues [3], the numerous possible combinations of which contribute to their diverse effects on chromatin structure and gene regulation (reviewed in [4]). A role for histone modifications in regulating long-term memory was first identified in the marine snail *Aplysia californica* [5]. The facilitating neuromodulator serotonin and the inhibitory modulator L-phenylalanyl-L-methionyl-L-arginyl-L-phenylalaninamide (FMRFamide) produced bidirectional effects on chromatin structure – histone acetylation and de-acetylation of the cAMP response element-binding protein (CREB) promoter – that led to opposing effects on gene expression, synaptic strength, and long-term memory (Figure 1). Subsequent studies throughout the past decade have revealed a conserved role for histone acetylation in long-term memory in mammals as well (reviewed in detail in [6]). Furthermore, in recent years, chromatin remodeling through histone deacetylase (HDAC) inhibitors has been found to reverse memory deficits as seen in aged mice [7], and in a mouse model with significant brain atrophy, induced by temporally and spatially restricted neuronal and synaptic loss [8]. In addition to histone acetylation, other histone modifications such as histone phosphorylation [9] and histone methylation [10] have also been shown to affect long-term memory.

Although histone modifications have an important role in memory formation, several challenges to their role in consolidation and maintenance of memories have been raised. Because the compaction of chromatin at one locus may be affected by modifications on remote histones, histone modifications are thought not to produce precise, locally specific changes in gene expression. Furthermore, marks are often reversed on short time scales, raising questions as to their role in maintaining long-term changes in behavior. However, recent studies reveal that histone modifications in the cortex can be critical for long-term memory consolidation. For olfactory associative memory in mice, an early cortical tag is required consisting of increased H3 histone acetylation that assumes the subsequent hippocampal–cortical interactions necessary for maintenance and storage of long-term memory [11]. In addition, a series of histone modifications, including histone phosphorylation and trimethylation, although only transiently activated in the hippocampus, have a more delayed but persistent role in the cortex, which is necessary for long-term memory consolidation [12]. These studies develop the idea that chromatin modifications, including their kinetics and duration of action, vary by region, and suggest that epigenetic mechanisms may in certain instances serve as a gateway for remote memory consolidation and maintenance.

DNA methylation in memory storage

More recently, DNA methylation has emerged as an important regulator of memory. This new emphasis can be traced to the discovery of several DNA methyltransferase (DNMT) enzymes that are differentially expressed in neurons, two of which, DNMT 1 and 3a, show expression in adulthood [13]. The DNMTs function in both *de novo* methylation (DNMT 3a and 3b) and maintenance methylation (DNMT1). They almost exclusively mark the 5′ position of cytosine residues followed by guanine, known as cytosine–phosphate–guanine (CpG) sites, which are statistically over-represented at promoter regions in dense clusters known as CpG islands. Methylation here almost always results in transcriptional repression, either by inhibiting transcription factor binding or recruiting methyl-binding domain proteins (MBDs)

and histone de-acetylases for long-term silencing of gene expression.

Initial studies on the effects of early-life experience on later-life cognition implicated DNA methylation in the persistence of certain types of emotional memories, even showing inheritance of behaviors for one or more generations. Rat pups (*Rattus norvegicus*) deprived of proper maternal care such as licking, grooming, and ached-back nursing were similarly abusive to their own offspring, and this was linked to persistent changes in DNA methylation of the glucocorticoid receptor gene in the hippocampus [14]. The epigenetic marks were likewise heritable and only partially reversed by cross-fostering with a nurturing mother, suggesting that behavior is driven by heritable changes in the epigenome, not the postnatal environment. Subsequent work also demonstrated that abusive behavior is at least partially reversible later in life through reversal of the epigenetic changes alone [15]. Childhood abuse shows similar effects on the epigenetic programming of the glucocorticoid receptor in humans. Suicide victims with a history of childhood abuse exhibit decreased glucocorticoid receptor mRNA levels corresponding with a high degree of methylation at its promoter, compared with suicide victims with no history of childhood abuse [16].

Recently a focus on activity-dependent epigenetic changes at individual loci linked with causally related electrophysiological and behavioral consequences has provided more direct indications of DNA methylation's role in memory storage. In response to fear conditioning, it was observed that the gene protein phosphatase 1 (*PP1*), which suppresses memory formation, was transcriptionally inhibited by methylation at specific sites in its promoter, whereas the gene *reelin*, which promotes memory formation, was rapidly induced by demethylation at its promoter [17]. These events together led to the coordinated enhancement of long-term potentiation (LTP), associative learning, and fear memory in mice. Although methylation at both of these gene loci in the hippocampus were found to return to baseline levels within 24 h [17], another study found that methylation of the calcineurin (*CaN*) gene in the prefrontal cortex during memory storage lasted up to 30 days [18], suggesting that hippocampal DNA methylation may serve a more transient function, but that cortical methylation may be necessary for the maintenance of long-term memories. These studies again underscore the fact that kinetics, duration, and readout of epigenetic modifications will vary in different brain regions, cell types, and genes. In another series of experiments, DNA methylation was found to be not only persistent, but also highly specific and dynamic in the regulation of memory-related genes. Methylation at the *Bdnf* gene was found to regulate alternative splicing in the production of brain-derived neurotrophic factor (BDNF) isoforms with specific consequences on memory storage [19]. The alternatively spliced exons have distinct promoters, which can be independently regulated, allowing for nuanced and highly coordinated gene regulation (Figure 2). Next-generation sequencing with single-nucleotide resolution [20] promises to offer genome-wide analysis linking methylation status, gene expression, and behavior.

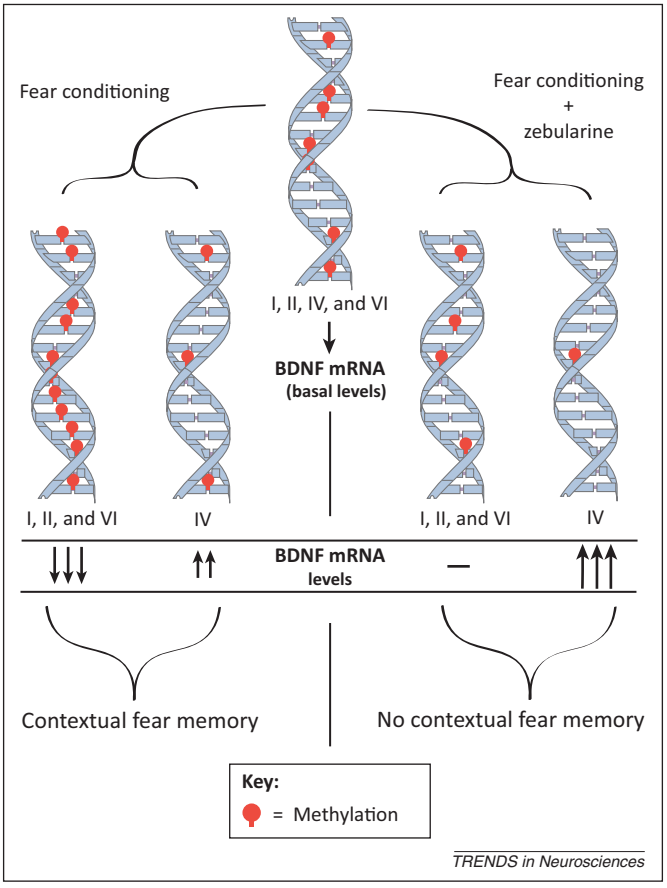

Figure 2. High specificity in the regulation of a single gene in memory formation [19]. Lubin *et al.* elegantly show how chromatin changes can lead to highly specific effects at a given gene locus, allowing for coordinated control over the expression of individual exons. The rat *Bdnf* gene has four alternatively spliced 5′ noncoding exons (I, II, IV, and VI), governed by distinct promoters, in addition to a single 3′ coding exon (IX). It is believed that the noncoding exons regulate temporal and spatial expression of the BDNF protein. In the steady state, the primers for exons I, II, IV, and VI of the *Bdnf* gene exist in partially methylated states. Fear conditioning results in heavy methylation of exons I, II, and VI whose mRNA levels fall dramatically, whereas exon IV undergoes significant demethylation and subsequent increase in mRNA levels. These changes in expression are important for contextual fear memory. Application of zebularine, an inhibitor of methylation, along with fear conditioning produces minimal demethylation at exons I, II, and VI with little accompanying change in mRNA and extensive demethylation of exon IV leading to a very robust increase in mRNA. As a result, no contextual fear memory is formed. Abbreviation: BDNF, brain-derived neurotrophic factor.

Noncoding RNAs in epigenetic and transcriptional regulation

Although growing evidence indicates that epigenetic changes in adult neurons are necessary for long-term information storage, the mechanisms that give rise to activity-dependent epigenetic changes remain poorly understood. How is DNMT recruited to specific regions when it lacks sequence specificity beyond CpG [21]? How does fear conditioning lead to differential methylation of distinct promoters in a single gene for *Bdnf*? In short, how does the cell 'know' which genes to tag for expression, which to silence? This remains poorly understood, but promising discoveries in the world of small RNAs may constitute a first step.

The recent discovery of several classes of small noncoding RNAs with nuclear expression and functional roles in targeting gene promoters for epigenetic regulation has underscored the important role of RNAs as regulators of gene expression in addition to the well-described function

of microRNAs in post-transcriptional regulation of cytoplasmic mRNAs. The idea that RNAs could provide the key to specificity in gene regulation is not new. In 1969, almost 30 years before the discovery of miRNAs or even protein transcription factors, an RNA-driven model was proposed [22], developing an earlier suggestion [23] that a complementary strand of RNA could form a sequence-specific complex with genomic DNA to effect transcriptional regulation [22]. However, it was not until decades later that widespread evidence for RNA-dependent transcriptional regulation was uncovered through the discovery and characterization of small noncoding RNAs.

The first class of small noncoding RNAs to be identified was the microRNAs [24,25]. These are 19–22 nucleotide small RNAs that have some function as transcriptional regulators in plants, but function entirely as post-transcriptional regulators of gene expression in animals. Initial excitement over the burgeoning field of miRNAs came from the revelation of a vast and unexplored set of conserved small RNA genes whose use of shared RNAi-machinery facilitated gene regulation with unprecedented specificity and complexity. The large-scale identification of miRNAs [26–28], and the development of tools to mine and characterize them, led to the discovery of several other classes of small RNAs, many of which had nuclear functions. Endogenous siRNAs (endo-siRNAs) serve various repressive roles including assembly of heterochromatin, silencing of retrotransposons, and inhibition of RNA polymerase II [29–31]. Furthermore, a host of newly characterized small RNAs participate in almost every aspect of nuclear mRNA processing and regulation, from transcription initiation (transcription initiation RNAs, tiRNAs) to splicing (splice-site RNAs, spliRNAs) to transcriptional silencing (repeat associated RNAs, RASiRNAs) [32].

Finally, a recently discovered class of slightly larger 26- to 32-nucleotide small RNAs, known as piRNAs, which were initially thought to be germline specific [33,34], were linked to transcriptional regulation via *de novo* DNA methylation. piRNAs are characteristically generated from long genomic clusters, sometimes larger than 100 000 bases, with almost all piRNAs within a cluster being derived in the same orientation, suggesting that piRNAs are transcribed en masse in long precursor RNA strands. The discovery of an upstream promoter common to piRNAs in *Caenorhabditis elegans* [35], together with a functional forkhead transcription factor that can bind and activate these promoters [36], has provided a possible mechanism for how piRNAs are generated. Importantly, piRNA clusters are not necessarily sequence conserved across species, but are highly syntenic, in that their relative locations across genomes are conserved [37], suggesting that their location of origin is crucial to their function. Initial discoveries that invertebrate piRNAs, such as those in *C. elegans* and *Drosophila*, are generated from repeat and transposon-rich regions of the genome, suggested that piRNAs function in transposon silencing. For example, piRNAs generated antisense to transposons at the same locus could facilitate base-pairing-mediated cleavage and silencing of the target transposons. Further elaboration of a 'ping-pong' mechanism of piRNA biogenesis proposed that not only do primary piRNAs cleave target transposons, but in that process, they could generate secondary piRNAs that are capable of cleaving the original piRNA cluster at a defined offset, generating still further piRNAs and thus creating an amplification cycle to ensure persistent production of piRNAs at a given locus. Although an attractive hypothesis, only a small fraction of piRNAs appear to be generated in this manner, suggesting other mechanisms may be at play. In addition to their biogenesis (for further review see [38]), the function of many piRNAs also remains enigmatic, but progress has been made in recent years. piRNA associate functionally with piwi, a member of the argonaute family of proteins, of which some homologs are nuclear and some cytoplasmic. Functional loss of piwi protein activity and mutations in the piRNA biogenesis pathway have consistently led to phenotypes of infertility and sterility in worm, fly, zebrafish, and mice [39]. Initial observations that invertebrate piRNAs are generated from transposon-rich areas of the genome suggested that piRNAs are positioned not only to directly cleave antisense transposon transcripts, but also to recruit epigenetic factors at the same transcriptional locus for transposon silencing. For instance, mice lacking one or more of their piwi homologs were found to have substantial demethylation and derepression of transposable elements targeted by germline piRNAs [40–43]. These observations pointed toward a role for piRNAs in maintaining germline genome integrity and tranposon control through *de novo* DNA methylation. However, the large majority of piRNAs in mammals derive from nontransposon associated, gene-rich, regions of the genome [44], and a few even from individual mRNA transcripts [45,46], suggesting broader functions for piRNA beyond transposon silencing, including the regulation of gene expression. Although piRNAs were initially thought to be restricted in expression to germ line and stem cells, a broader role for piRNAs is further suggested by the discovery of piRNAs in somatic cells [47–49]. Furthermore, piRNAs were recently found in high abundance in neurons of the *Aplysia* central nervous system (CNS), where at least one piRNA was shown to have a critical role in the epigenetic regulation of synaptic plasticity during long-term memory storage [50]. It is to this example that we now turn.

A role for piRNAs in the epigenetic control of memory

The search for small RNAs involved in learning and memory in *Aplysia* initially focused on the functional analysis of two highly abundant and brain-specific miRNAs in *Aplysia*, miR-124 and miR-22, which, along with several other miRNAs, undergo a transient, but significant decrease in response to serotonin treatment. miR-124 was determined to act as a post-transcriptional inhibitory constraint on the memory-related transcription factor CREB-1 [51], and miR-22 as an inhibitory constraint on the prion-like translation factor cytoplasmic polyadenylation element binding protein (CPEB) (P. Rajasethupathy and F. Fiumara, personal communication). As a result, the serotonin-induced de-repression of these miRNA targets led to, and provided a mechanism for, the enhancement of different components of synaptic plasticity underlying long-term memory. In the process of studying these miRNAs, the researchers serendipitously discovered a highly

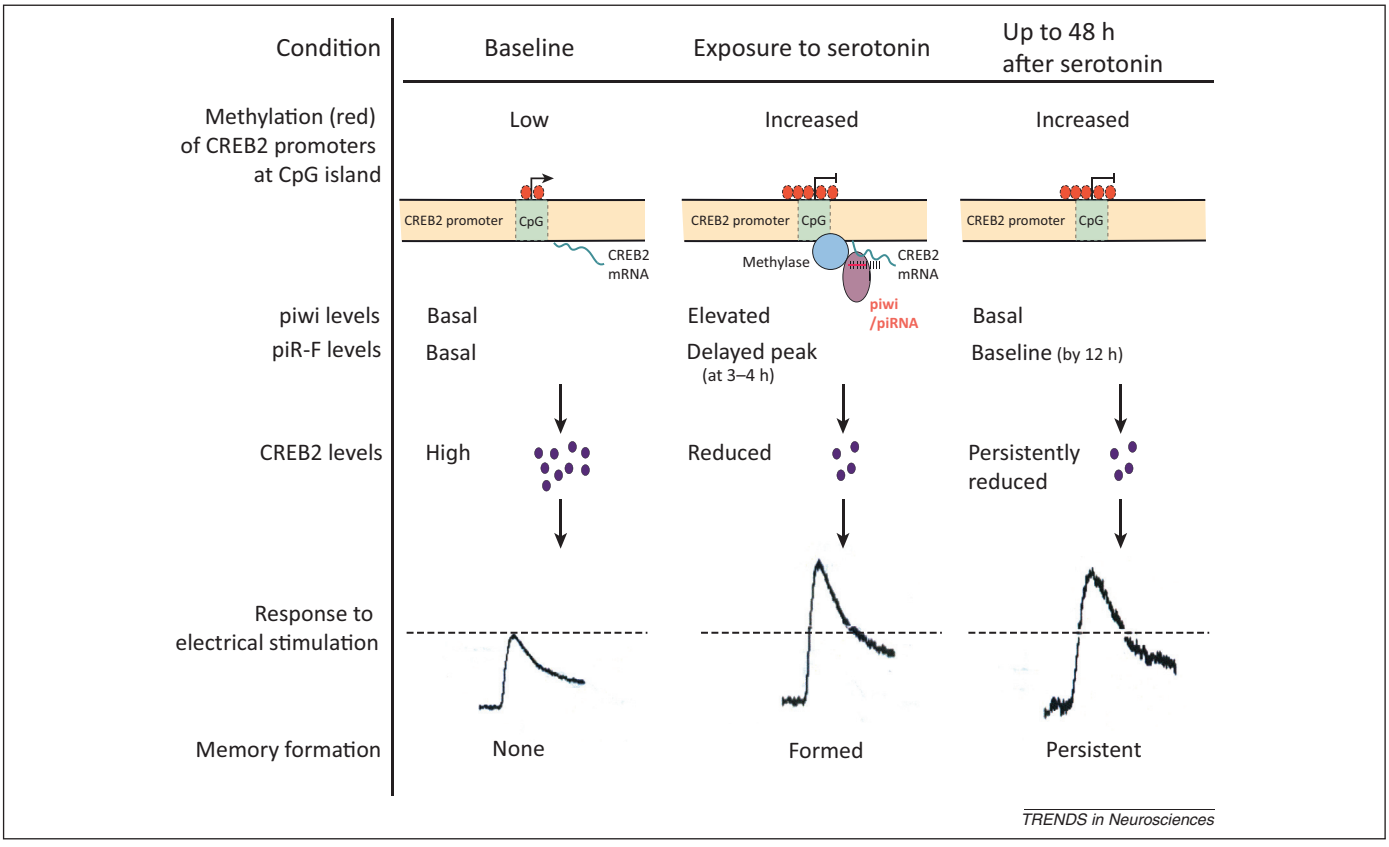

Figure 3. An *Aplysia* piRNA involved in the serotonin-dependent epigenetic regulation of gene expression underlying long-term memory [50]. In *Aplysia* neurons, Rajasethupathy *et al.* showed piwi/piRNA-dependent methylation at the CREB2 promoter in response to serotonin, which led to long-lasting depression of CREB2 levels and the persistence of synaptic facilitation. In the baseline condition, low methylation at the proximal CpG site of the CREB2 promoter allows high production of CREB2 and the absence of long-term facilitation. Exposure to serotonin causes a significant but delayed upregulation of the neuronally enriched piRNA piR-F, which recruits the piwi/piRNA complex to bind a target site on the nascent transcript in the 5′-UTR of CREB2 pre-mRNA by putative sequence-specific direction. This leads to the recruitment of factors that promote the activation of DNMT, a DNA methyltransferase. DNMT acts to methylate the proximal (but not the distal) CpG island in the CREB2 promoter, leading to reduced expression of CREB2, induction of long-term facilitation, and formation of memory. By 12 h, piR-F levels return to baseline, but up to 48 h later, methylation of the proximal CpG island persists, ensuring long-lasting depression of CREB2 levels and the persistence of long-term facilitation and memory. Adapted from the summary figure in [50]. Abbreviations: Piwi, P-element induced wimpy testis; piR-/piRNA, piwi-interacting RNA; CREB-1,2, cAMP response element-binding protein-1,2; DNMT, DNA methyltransferase.

abundant and nuclear-localized class of 28-nucleotide RNAs in brain that function in the epigenetic regulation of long-term memory [50]. These novel, neuronally-expressed, small RNAs were found to be piRNAs by several stringent criteria including the requirement for a clustered arrangement in the genome, characteristic 2′-*O*-methyl modification at their 3′ ends, and selective coprecipitation with the neuronally-expressed piRNA carrier protein piwi but not with the miRNA carrier argonaute. Furthermore, piwi knockdown led to specific depletion of these piRNAs. Functional analyses revealed that a subset of the piRNAs in *Aplysia* CNS was upregulated by serotonin. Importantly, and consistent with known roles of piRNAs in DNA methylation in germline and stem cells, one particular piRNA, piR-F, specifically upregulated by serotonin, was found to cause increased piwi/piRNA-dependent methylation at the promoter of inhibitory transcription factor CREB2, which corresponded to a long-term (up to 48 h) drop in CREB2 transcript and protein levels and an enhancement in facilitation of synapses (Figure 3). These effects on CREB2 and synaptic facilitation were reversed by inhibition of piwi by antisense oligonucleotides or DNA methyltransferase by *N*-phthalyl-L-tryptophan (RG108). These data provided a piRNA-dependent epigenetic mechanism by which transient stimuli can cause more persistent changes in the function of neurons involved in memory storage.

The discovery that piRNAs exist outside the germline, in several major organs of *Aplysia*, particularly the nervous system, suggests much broader roles for piRNAs than previously appreciated. It is also interesting to note that piRNAs in *Aplysia* appear to have opposing functions from previously studied miRNAs. For instance, although miRNAs in *Aplysia* CNS turn over rapidly in response to serotonin, the opposite holds for piRNAs. Many of the abundant piRNAs slowly and more persistently increase with exposure to serotonin. Although relatively few small RNAs have thus far been studied in *Aplysia*, the data suggests that miRNAs and piRNAs have bidirectional effects on synaptic facilitation. Although miRNAs constrain serotonin dependent long-term facilitation, piwi/piRNAs enhance it. Finally, miRNAs and piRNAs appear to have distinct targets. Although miRNAs target facilitators of synaptic plasticity (e.g., CREB1, CPEB), the piRNAs target repressors (e.g., CREB2). On the basis of these observations, a working model was proposed [50] of small RNA regulation in the *Aplysia* nervous system wherein two distinct classes of small RNAs are bidirectionally regulated by serotonin, acting in a coordinated

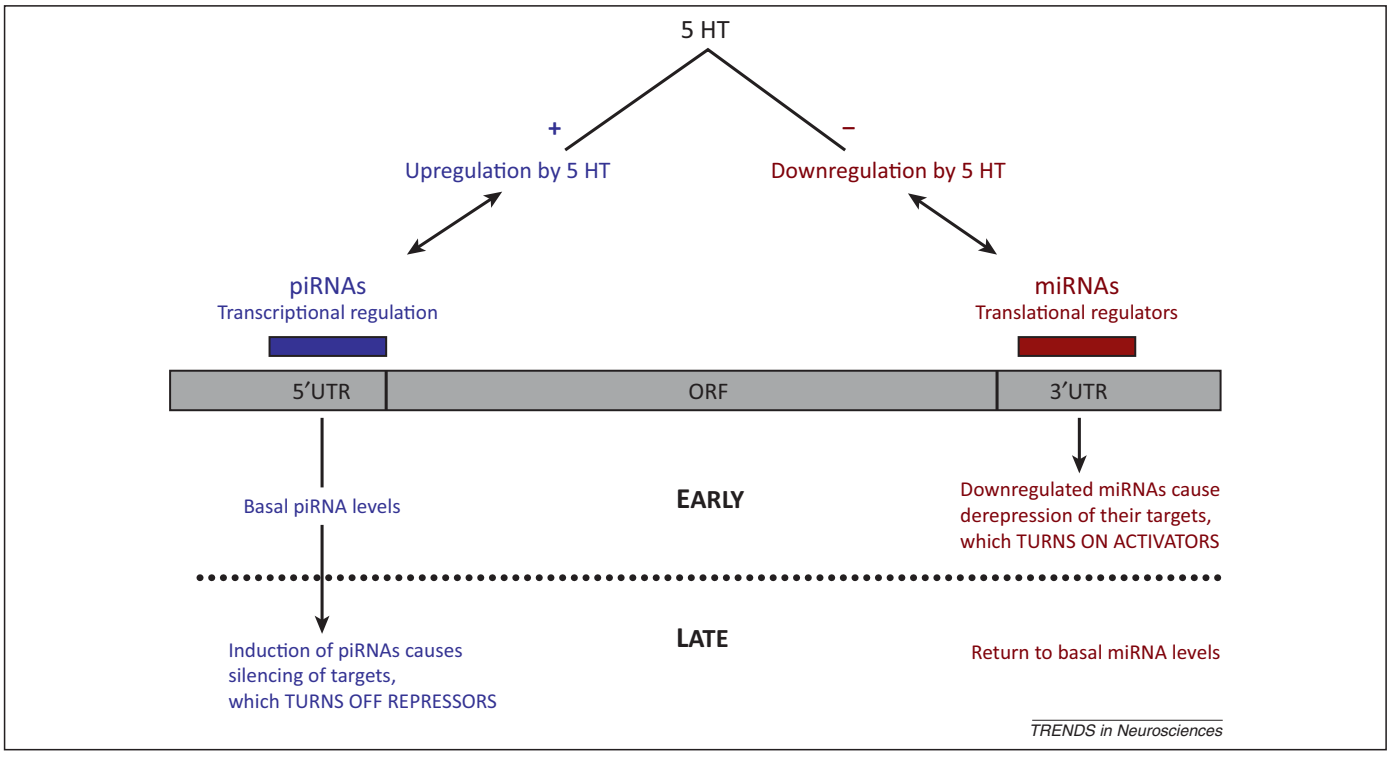

Figure 4. A working model: the integrative action of small RNAs during synaptic plasticity [50]. A model of bidirectional small RNA function during synaptic plasticity in *Aplysia*: piRNAs and miRNAs are transcriptional and translational repressors at baseline. With exposure to serotonin, piRNAs become activated, which leads to further transcriptional repression of its targets (inhibitors of synaptic facilitation) and the eventual enhancement of synaptic strength. By contrast, serotonin downregulates miRNAs, which leads to translational upregulation of its targets (enhancers of synaptic facilitation) and the eventual enhancement of synaptic strength. The two classes of small RNAs in *Aplysia*, therefore, may act in a coordinated fashion to regulate long-term memory processes. Abbreviations: 5HT, 5-hydroxytryptamine or serotonin; miRNA, microRNA; piRNA, piwi-interacting RNA; UTR, untranslated region.

fashion on a functionally segregated population of targets, to enable memory related synaptic plasticity (Figure 4).

Recent work provides evidence that the piRNA pathway is critically involved in initiating multigenerational epigenetic memory in the *C. elegans* germline, lasting up to 20 generations. Once triggered, memory maintenance becomes independent of piRNAs, and thereafter depends only on the chromatin machinery [52,53]. Although these studies provide evidence of transgenerational silencing of transposons and foreign genes, they suggest that piRNA regulation of endogenous genes in other cell types such as neurons, may have similarly long-lasting effects that one might expect for memory storage. In addition, the activity of long interspersed nuclear element (LINE-1) retrotransposons, known to be regulated by piwi/piRNAs [54], was found to influence experience-dependent neuronal plasticity in the mouse hippocampus [55,56]. These findings suggest that the piRNA pathway may be integral to neuronal plasticity underlying memory storage not only in *Aplysia*, but also in the mammalian hippocampus, through regulation of retrotransposons, plasticity-related genes, or both.

Concluding remarks

Memory – an emergent property of neurons that arises from recursive communication between genes and synapses – is a powerful adaptive tool. Epigenetic modifications provide a mechanism for transient stimuli to be translated into more stable and potentially long-lasting internal representations within the cell, through persistent changes in gene expression. Although it is not surprising that epigenetic mechanisms evolved early for use in cellular communication and information storage, it is noteworthy that at least some of these mechanisms have been highly conserved, from slime molds to humans.

DNA methylation, in particular, has several adaptive advantages for information storage: the effects are long lasting, activity dependent, and highly specific. From the earliest studies on gene imprinting to more recent studies on cell fate determination and transgenerational inheritance, the lasting effects of DNA methylation in response to transient stimuli have been well established. Furthermore, although we have long known that epigenetic changes can be developmentally regulated, more recent findings expand this view to include changes induced by neurotrophins, neurotransmitters, and synaptic activity. Finally, the effects of DNA methylation can be localized to a single gene, or even single exon within a gene, thereby effectively modulating long-term changes in gene expression in an isoform and cell type-specific way. This level of specificity provides reason to believe that a neuron's epigenome may contain clues about its experiential history.

Although the collection of studies presented in this review provides support for the notion that long-term memory can be governed by stable epigenetic changes, the role of DNA methylation in neuronal plasticity of differentiated cells in the adult brain has been met with some skepticism. One primary concern is that methylation is appropriate for changes in gene expression that are permanent, as is the case during development; however,

Box 1. Outstanding questions

- How does the epigenome of individual neurons in a neural network change during learning and memory?
- Are the observed changes in histone modification, and especially DNA methylation, at individual loci causal rather than correlated to observed deficits in long-term memory storage?
- What are the kinetics of epigenetic changes in neurons as a memory evolves or persists? Do epigenetic modifications behave like switches (as we assume) or confer graded responses?
- What are the mechanisms that drive experience-dependent epigenetic changes in neurons? Could this occur through piRNAs, or any other type of small RNA, in other systems as well?

New tools may yield new insights

- Single cell epigenomic and transcriptomic technology for monitoring individual neurons in an evolving memory network.
- Transgenic mice containing single nucleotide loss-of-function mutations that provide functional causality.
- Large scale crosslinking and immunoprecipitation (CLIP) analysis linked to highthroughput sequencing to further delineate underlying small RNA- and protein-dependent mechanisms that translate activity into epigenetic changes.

this may not be the case in adult neurons because the plastic nature of synaptic connections, by definition, requires bidirectional and reversible changes in gene expression. A possible explanation is that neurons may use a high activity threshold such that only very strong stimuli lead to lasting epigenetic changes in neurons capable of encoding a persistent memory. Additionally, the recent identification of functional DNA demethylases in neurons of the hippocampus suggests some level of reversibility, which may accommodate both endurance and plasticity of neuronal connections depending on context. Finally, it may be the case that not all neurons in a newly created memory trace are subject to epigenetic change, but rather the few neurons that do may initiate a cell wide form of plasticity that allows global synaptic strengthening. In this way, certain neurons in the memory trace become memory hubs, with extensive connectivity to other neurons in the same trace, providing a mechanism for binding and retrieval of that memory. Future studies may help clarify these issues (Box 1).

As we consider the progress in the field of learning and memory over the last several decades, it is interesting to note that there have been many molecular candidates with 'information storage' capacity. Second messengers such as cAMP were intriguing because they could transduce a very transient calcium influx into a longer-lasting cellular signal by dispersing the information to many downstream signals [57]. Then kinases with some level of persistent activity were discovered: protein kinase A (PKA) [58], and recently protein kinase Mζ (PKMζ) also [59], although this has been challenged [60,61], when independent of their regulatory domain, can prolong the magnitude and duration of catalytic activity; Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), when phosphorylated, becomes autocatalytic for an extended time even in the absence of calcium and calmodulin [62–64]; PKCα by anchoring to synaptic membranes, increases its effective concentration and local activity for a greater duration of time [65]. Long-term stable changes, however, need not occur through persistently active enzymes. Transcriptional switches (CREB1 and CREB2) [66,67], translational (prion-like) switches (CPEB) [68,69], and cytoskeletal switches (i.e., actin) [70] are perhaps even more effective in translating short-term events into long-term events. Each of these switches has some level of persistent activity but vary in their duration. Therefore, as a cell receives information, the initial responders to calcium transients begin a cascade of events where persistent molecules pass the baton to progressively slower and more robust switches, until eventually the final switch is read out through enzymatic, structural, or gene expression changes. Therefore, current models for the role of epigenetic mechanisms in memory are compatible with previous models where one of the switches, or perhaps the final responder in a cascade of switches, may be an epigenetic switch that leads to stable changes in gene expression underlying long-term memory.

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References

1 Shaulsky, G. and Kessin, R.H. (2007) The cold war of the social amoebae. *Curr. Biol.* 17, R684–R692
2 Sawarkar, R. *et al.* (2009) Histone deacetylases regulate multicellular development in the social amoeba *Dictyostelium discoideum*. *J. Mol. Biol.* 391, 833–848
3 Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science* 293, 1074–1080
4 Day, J.J. and Sweat, J.D. (2011) Epigenetic mechanisms in cognition. *Neuron* 70, 813–829
5 Guan, Z. *et al.* (2002) Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell* 111, 483–493
6 Graff, J. and Tsai, L.H. (2013) Histone acetylation: molecular mnemonics on the chromatin. *Nat. Rev. Neurosci.* 14, 97–111
7 Peleg, S. *et al.* (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* 328, 753–756
8 Fischer, A. *et al.* (2007) Recovery of learning and memory is associated with chromatin remodelling. *Nature* 447, 178–182
9 Koshibu, K. *et al.* (2009) Protein phosphatase 1 regulates the histone code for long-term memory. *J. Neurosci.* 29, 13079–13089
10 Gupta, S. *et al.* (2010) Histone methylation regulates memory formation. *J. Neurosci.* 30, 3589–3599
11 Lesburguères, E. *et al.* (2011) Early tagging of cortical networks is required for the formation of enduring associative memory. *Science* 331, 924–928
12 Graff, J. *et al.* (2012) Dynamic histone marks in the hippocampus and cortex facilitate memory consolidation. *Nat. Commun.* 3, 991
13 Goto, K. *et al.* (1994) Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation* 56, 39–44
14 Weaver, I.C. *et al.* (2004) Epigenetic programming by maternal behavior. *Nat. Neurosci.* 7, 847–854
15 Weaver, I.C. *et al.* (2005) Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. *J. Neurosci.* 25, 11045–11054
16 McGowan, P.O. *et al.* (2009) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat. Neurosci.* 12, 342–348
17 Miller, C.A. and Sweatt, J.D. (2007) Covalent modification of DNA regulates memory formation. *Neuron* 53, 857–869
18 Miller, C.A. *et al.* (2010) Cortical DNA methylation maintains remote memory. *Nat. Neurosci.* 13, 664–666

19 Lubin, F.D. *et al.* (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *J. Neurosci.* 28, 10576–10586
20 Guo, J.U. *et al.* (2011) Neuronal activity modifies the DNA methylation landscape in the adult brain. *Nat. Neurosci.* 14, 1345–1351
21 Yu, N.K. *et al.* (2011) DNA methylation-mediated control of learning and memory. *Mol. Brain* 4, 5
22 Britten, R.J. and Davidson, E.H. (1969) Gene regulation for higher cells: a theory. *Science* 165, 349–357
23 Crick, F.H. (1958) On protein synthesis. *Symp. Soc. Exp. Biol.* 12, 138–163
24 Lee, R.C. *et al.* (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complimentarity to lin-14. *Cell* 75, 843–854
25 Wightman, B. *et al.* (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* 75, 855–862
26 Lagos-Quintana, M. *et al.* (2001) Identification of novel genes coding for small expressed RNAs. *Science* 294, 853–858
27 Lau, N.C. *et al.* (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* 294, 858–862
28 Lee, R.C. and Ambros, V. (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* 294, 862–864
29 Verdel, A. and Moazed, D. (2005) RNAi-directed assembly of heterochromatin in fission yeast. *FEBS Lett.* 579, 5872–5878
30 Ghildiyal, M. *et al.* (2008) Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. *Science* 320, 1077–1081
31 Guang, S. *et al.* (2010) Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. *Nature* 465, 1097–1101
32 Taft, R.J. *et al.* (2010) Nuclear-localized tiny RNAs are associated with transcription initiation and splice sites in metazoans. *Nat. Struct. Mol. Biol.* 17, 1030–1034
33 Aravin, A. *et al.* (2006) A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* 442, 203–207
34 Girard, A. *et al.* (2006) A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* 442, 199–202
35 Batista, P.J. *et al.* (2008) PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in *C. elegans*. *Mol. Cell* 31, 67–78
36 Cecere, G. *et al.* (2012) Promoters recognized by forkhead proteins exist for individual 21U-RNAs. *Mol. Cell* 47, 743–745
37 Farazi, T.A. *et al.* (2008) The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. *Development* 135, 1201–1214
38 Ishizu, H. *et al.* (2012) Biology of PIWI-interacting RNAs: new insights into biogenesis and function inside and outside of germlines. *Genes Dev.* 26, 2361–2373
39 Thomson, T. and Lin, H. (2009) The biogenesis and function of PIWI proteins and piRNAs: progress and prospect. *Annu. Rev. Cell Dev. Biol.* 25, 355–376
40 Aravin, A.A. *et al.* (2008) A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol. Cell* 31, 785–799
41 Brennecke, J. *et al.* (2008) An epigenetic role for maternally inherited piRNAs in transposon silencing. *Science* 322, 1387–1392
42 Kuramochi-Miyagawa, S. *et al.* (2008) DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev.* 22, 908–917
43 Watanabe, T. *et al.* (2011) Role for piRNAs and noncoding RNA in de novo DNA methylation of the imprinted mouse Rasgrf1 locus. *Science* 332, 848–852
44 Betel, D. *et al.* (2007) Computational analysis of mouse piRNA sequence and biogenesis. *PLoS Comp. Biol.* 3, e222
45 Robine, N. *et al.* (2009) A broadly conserved pathway generates 3′UTR-directed primary piRNAs. *Curr. Biol.* 19, 2066–2076
46 Saito, K. *et al.* (2009) A regulatory circuit for piwi by the large Maf gene traffic jam in *Drosophila*. *Nature* 461, 1296–1299
47 Brower-Toland, B. *et al.* (2007) *Drosophila* PIWI associates with chromatin and interacts directly with HP1a. *Genes Dev.* 21, 2300–2311
48 Malone, C.D. *et al.* (2009) Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. *Cell* 137, 522–535
49 Yan, Z. *et al.* (2011) Widespread expression of piRNA-like molecules in somatic tissues. *Nucleic Acids Res.* 39, 6596–6607
50 Rajasethupathy, P. *et al.* (2012) A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. *Cell* 149, 693–707
51 Rajasethupathy, P. *et al.* (2009) Characterization of small RNAs in *Aplysia* reveals a role for miR-124 in constraining synaptic plasticity through CREB. *Neuron* 63, 803–817
52 Shirayama, M. *et al.* (2012) piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* 150, 65–77
53 Ashe, A. *et al.* (2012) piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* 150, 88–99
54 Siomi, M.C. *et al.* (2010) How does the royal family of Tudor rule the PIWI-interacting RNA pathway? *Genes Dev.* 24, 636–646
55 Coufal, N.G. *et al.* (2009) L1 retrotransposition in human neural progenitor cells. *Nature* 460, 1127–1131
56 Muotri, A.R. *et al.* (2010) L1 retrotransposition in neurons is modulated by MeCP2. *Nature* 468, 443–446
57 Brunelli, M. *et al.* (1976) Synaptic facilitation and behavioral sensitization in *Aplysia*: possible role of serotonin and cyclic AMP. *Science* 194, 1178–1181
58 Castellucci, V.F. *et al.* (1980) Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase simulates facilitation of transmitter release underlying behavioral sensitization in *Aplysia*. *Proc. Natl. Acad. Sci. U.S.A.* 77, 7492–7496
59 Serrano, P. *et al.* (2008) PKMzeta maintains spatial, instrumental, and classically conditioned long-term memories. *PLoS Biol.* 6, 2698–2706
60 Volk, L.J. *et al.* (2013) PKM-ζ is not required for hippocampal synaptic plasticity, learning and memory. *Nature* 493, 420–423
61 Lee, A.M. *et al.* (2013) Prkcz null mice show normal learning and memory. *Nature* 493, 416–419
62 Lisman, J.E. (1985) A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. *Proc. Natl. Acad. Sci. U.S.A.* 82, 3055–3057
63 Miller, S.G. *et al.* (1986) Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: a Ca^{2+}-triggered molecular switch. *Cell* 44, 861–870
64 Malinow, R. *et al.* (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* 245, 862–866
65 Staudinger, J. *et al.* (1997) Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-α. *J. Biol. Chem.* 272, 32019–32024
66 Dash, P.K. *et al.* (1990) Injection of cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. *Nature* 345, 718–721
67 Bartsch, D. *et al.* (1995) *Aplysia* CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. *Cell* 83, 979–992
68 Si, K. *et al.* (2003) A neuronal isoform of the *Aplysia* CPEB has prion-like properties. *Cell* 115, 879–891
69 Si, K. *et al.* (2003) A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in *Aplysia*. *Cell* 115, 893–904
70 Okamoto, K.I. *et al.* (2004) Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity. *Nat. Neurosci.* 7, 1104–1112