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Review

DNA methylation: dynamic and stable regulation of memory

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Abstract

Epigenetic mechanisms have emerged as a central process in learning and memory. Histone modifications and DNA methylation are epigenetic events that can mediate gene transcription. Interesting features of these epigenetic changes are their transient and long lasting potential. Recent advances in neuroscience suggest that DNA methylation is both dynamic and stable, mediating the formation and maintenance of memory. In this review, we will further illustrate the recent hypothesis that DNA methylation participates in the transcriptional regulation necessary for memory.

Keywords: behavior; brain; DNA methylation; epigenetics; learning; memory.

Introduction

The brain is a highly plastic structure, which is able to adjust rapidly and repeatedly to changes in the environment. Learning, which results from the association of two initially unrelated inputs, is an excellent illustration of the brain's dynamic capacity to respond to the environment. For example, touching a hot burner on the stove leads to a learned association between pain and the black coils on the stovetop. Paradigms used to study memory in rodents employ similar types of associative conditioning in which a specific context or cue (e.g., novel environment or auditory tone) is paired with a previously independent stimulus (i.e., foot shock or food reward). Different brain regions are involved in the associative process, supporting learning. The acquired information will subsequently be stored as memory so that it can be recalled in the presence of the environmental stimulus that triggered the initial learning. Retrieval of this memory will subsequently modify behavior (e.g., avoid touching the stove). The formation and consolidation of many types of memories occur shortly after learning in the hippocampus, after which many are subsequently incorporated into and maintained in cortical areas. Interestingly, in the absence of the reinforcing environmental stimulus a memory can be extinguished. We would for example eventually lose our fear of touching a burner on a broken stove that is incapable of heating. This ability to modify established memories further demonstrates the brain's capacity for plastic changes. At the cellular level, memory requires a carefully coordinated program of transcription and translation, in addition to structural and functional changes in the brain (Figure 1). Memory results from the environments transcriptional impact on our genome suggesting that epigenetics could be a critical regulator of this complex cognitive process.

Epigenetics has traditionally been viewed as changes that are inherited through generations or cell division and are not dependent on the DNA sequence itself (1). However, numerous examples of intrinsic reversibility of epigenetic changes have been established (2, 3). In light of this and due to their critical involvement in gene regulation epigenetic mechanisms represent an attractive and reversible means for the brain to respond and adapt to specific environmental changes. In addition, epigenetic alterations triggered by external factors and environmental stimuli can lead to molecular responses and enduring changes in gene expression. Therefore, transient activation of epigenetic players can contribute to heritable gene regulation constituting cellular memory (4, 5). Recent evidence suggests that epigenetic events, such as histone modifications and DNA methylation occur in post-mitotic cells in the brain to regulate key cellular processes involved in learning and memory.

DNA in living cells is not naked. It wraps around core histone proteins to form nucleosomes, which subsequently becomes a highly organized structure termed chromatin. Histone tails extend from the surface of the nucleosome, where several post-translational modifications including acetylation and methylation have been identified (6, 7). Combinations of different histone modifications on the tails participate in the regulation of chromatin structure, controlling access to genes for transcription (8–10). This combinatorial nature of different histone marks adds a layer of complexity to predicting transcriptional outcome based on the observation of a single post-translational modification. Histone acetylation and phosphorylation have been associated with an open chromatin state and active transcription, whereas histone methylation can mediate active and silent chromatin states. DNA itself can also be directly modified through the covalent addition of a methyl group ($-CH_3$) on the cytosine pyrimidine ring in DNA located in a CpG dinucleotide. DNA methylation can participate in transcriptional regulation and protein synthesis (11). Small stretches of DNA known as CpG islands are comparatively rich in CpGs and frequently free of DNA methylation. These CpG islands are

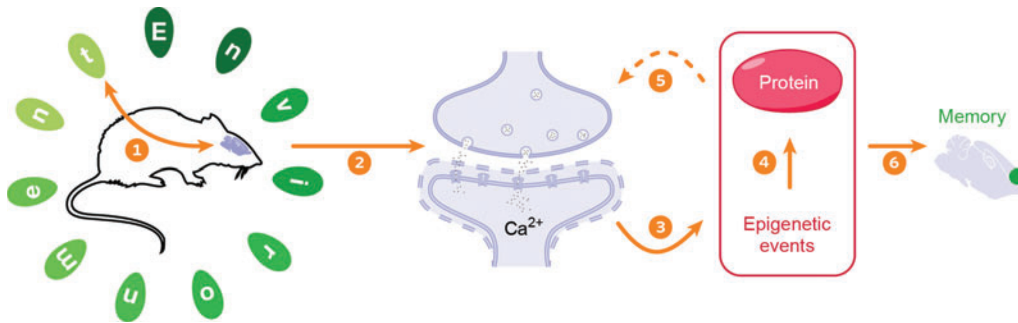

Figure 1 Outline of the basic structural and molecular events that take place during memory formation.
Associative learning can be induced by different environmental factors (1), which will lead to cellular changes in the brain in order to induce structural plasticity and increase synaptic strength (2). The transcriptional program is regulated in part by epigenetic events in order to dynamically modulate protein synthesis through transcriptional activation and silencing (3, 4) to establish long lasting memory (6). Thus, epigenetic changes can mediate and be mediated by behavioral changes, synaptic plasticity and structural changes (i.e., spine enlargement) to potentiate long-term memory processes (3, 5).

often located within the promoter regions of specific genes and DNA methylation within these islands undergoes dramatic changes during the early stages of embryonic development. DNA methylation marks are almost completely erased in the peri-implantation embryo and then methylation patterns are set up over the genome (12, 13). This epigenetic reprogramming is believed to be essential for cellular totipotency (14). During embryonic development, the *de novo* DNA methyltransferases DNMT3A and 3B are needed for reprogramming of DNA methylation. DNMT3A and DNMT3B establish the original DNA methylation patterns during early development whereas patterns of DNA methylation are maintained by DNMT1. Homozygous deletions of any of these genes in mice are lethal either before birth (*Dnmt1* and *Dnmt3b*) or shortly after (*Dnmt3a*) (15, 16). Controversial findings suggest that DNMT3A and 3B are also involved in both the addition and removal of methyl groups. The removal has been proposed to occur through deamination. However, the precise mechanism for DNA demethylation remains elusive (see 'DNA methylation and memory' Section) (17). Different families of proteins bind methylated DNA. These proteins can specifically recognize methylated DNA and act as mediators of the methylation signal recruiting DNMTs and co-repressors [e.g., histone deacetylases (HDACs) and histone methyltransferases (HMTs)]. The players that mediate the addition of a methyl mark, their removal and those that have the ability to bind methylated DNA have been implicated in cellular functions of the brain. This suggests that DNA methylation might participate in the transcriptional regulation necessary for memory supporting synaptic plasticity mechanisms and stable memory formation.

Writers and readers of DNA methylation identified in the brain

Recent evidence suggest that DNA methylation occurs in post-mitotic and mitotic cells, in which the balance and pattern of DNA methylation can be maintained by DNMTs and emerging candidates of DNA demethylation. The *de novo* (DNMT3A and 3B) and maintenance (DNMT1) DNA methyltransferases are expressed in the brain and have been implicated in the regulation of neuronal genes (18–23). Therefore, DNMT1 and DNMT3A could be participating in synaptic plasticity by establishing and maintaining a DNA methylation pattern that coordinates the expression of appropriate neuronal genes. Interestingly, the role of DNMT3A in the brain might be specific to brain regions and genomic locations because non-promoter methylation mediated by this methyltransferase can facilitate the transcription of neurogenic genes (24). Moreover, DNMT3a is also dynamically regulated in the nucleus accumbens (the brain's reward center) and influences behavioral response to cocaine use and stress (25). Surprisingly, the upstream signaling that controls DNMT-mediated regulation of transcription in the brain remains unclear.

Methyl binding proteins can also indirectly influence transcription. Ubiquitin-like, containing plant homeo domain (PHD) and really interesting new gene (RING) finger domains, 1 protein (UHRF1), a protein member of the Su(var)3-9, Enhancer-of-zeste, Trithorax (SET) and RING finger associated (SRA) domain family can bind to methylated DNA and mediate the maintenance of DNA methylation through the binding of hemimethylated DNA and recruitment of DNMT1 (26, 27). UHRF1 has been shown to be upregulated in fresh water snails retaining long-term memory (28). However, it remains to be addressed if this protein has a redundant function in the brain of higher model organisms where other methyl binding proteins have been implicated in cognitive function.

The methyl-CpG binding domain (MBD) family is comprised of well characterized proteins [methyl CpG binding protein 2 (MeCP2), MBD1, MBD2, MBD3 and MBD4] that are required to maintain neuronal function and homeostasis (29–31). The MBD proteins are capable of binding methylated DNA through the recognition of a single methylated CpG site (32). One of these members, MeCP2, is a critical player in the neurodevelopmental disease, Rett syndrome (33). MeCP2 interacts with methylated DNA and other proteins to

form a repressive complex that contributes to gene silencing. Interestingly, it could have other neuronal functions because MeCP2 can bind outside of a CpG island (34) and might function as a transcriptional activator through the binding of a distal methylated promoter (35). Despite its controversial function as a dual repressor and gene activator, MeCP2 appears to be participating in memory modulation through the regulation of important plasticity genes, such as brain-derived neurotrophic factor (*Bdnf*) (36, 37). Another MBD protein, MBD1, which is specific to heterochromatic regions (38) has been implicated in spatial learning and long-term synaptic potentiation (LTP) (39, 40). The ability of DNA methylation to regulate gene expression in post-mitotic neurons in addition to the importance of methyl-binding proteins in memory further suggests a role for DNA methylation in learning and memory.

Dynamic DNA methylation in the adult brain

DNA methylation changes observed in the brain are dynamic and bidirectional, with gain and loss of methylation observed throughout an organisms lifespan (41). These dynamic changes are likely to occur through a reciprocal relationship between DNA methylation and the environment. For example, the expression of DNMTs can modulate behavioral responses to drug abuse (25). Several reports suggest that the quality of early life environment can also impact adult behavior through changes to the epigenetic landscape, particularly DNA methylation (42–45). For instance, poor maternal care was associated with promoter methylation changes in the glucocorticoid receptor in pups. This epigenetic change persisted into adulthood and was associated with increased receptor expression (42) suggesting that changes in DNA methylation can dynamically respond to the environment not only during embryonic development but also postnatally.

DNA methylation in memory

Dynamic DNA methylation events have been reported in the adult brain, particularly in the hippocampus. During learning, synaptic activity at hippocampal neurons initiates several signaling cascades, with changes occurring locally at the synapse in addition to in the nucleus where transcriptional changes are made to plasticity-related proteins. These changes are supported, at least in part, by epigenetic modifications (46, 47). Surprisingly, beyond a dependence on one of the earliest steps in synaptic transmission, glutamate binding to the N-Methyl-D-aspartic acid receptor (NMDAR), the upstream players that regulate DNMTs during learning have not yet been established (48–50).

The proteins responsible for *de novo* DNA methylation, DNMT3A and 3B, are upregulated in the hippocampus following fear learning (51). In this hippocampus and NMDAR-dependent paradigm, known as contextual fear conditioning, rats are trained to associate mild foot shock with a novel context. Local inhibition of DNMTs within the hippocampus disrupts formation of this fear memory in addition to estrogens ability to enhance memory for a novel object (49, 51–53). Moreover, the *Dnmt1-Dnmt3a* double knockouts (but not single knockouts of either gene) show impairments in a form of spatial learning, indicating that DNMTs serve non-redundant functions during memory acquisition (23).

Within an hour of training for contextual fear, suppressors of memory are hypermethylated whereas memory promoters are hypomethylated (51). These modifications in methylation are associated with corresponding transcriptional changes and are consistent with the need for a program of transcriptional changes during memory acquisition that requires a balance of activation and suppression. The memory promoter, *Bdnf*, is induced in the hippocampus with learning and has been the attention of several epigenetic studies. *Bdnf* methylation and expression is dynamically regulated in the hippocampus in an NMDA receptor-dependent manner during memory formation (37, 49, 54–56). Together, these data indicate that gene specific DNA methylation in the hippocampus is capable of responding to the environment in a rapid and dynamic fashion, and is associated with synaptic activity and cognition.

Successful support of memory formation suggests the need for DNA methylation to be dynamically regulated in a bidirectional and parallel manner, mediating the silencing of memory repressors through their hypermethylation, while promoting the activation of memory enhancers through demethylation. Although DNA demethylation appears to be operational in mammalian cells (17, 51, 57–59), the precise mechanism has not yet been identified. In mitotic cells, demethylation is a passive process that occurs through rounds of DNA replication and can be triggered either by downregulation of DNMT1 or through its inhibition by DNMT inhibitors [e.g., 5-azacytidine (5-azaC)]. However, DNA demethylation in non-dividing cells of the adult brain suggests that an active process must exist. Several DNA demethylases have been proposed, including MBD2 (60). However, their activities have been challenged (61–63) and it is still not clear which protein could function as an active DNA demethylase (64). Yet the number of documented demethylation events is accumulating and several hypotheses have been raised with additional candidate mechanisms for demethylation (17, 61). As removal of methyl groups from cytosines is energetically unlikely, alternative pathways involving DNA glycosylases and deaminases have been proposed (65–67). Glycosylase activity in active demethylation is well established in plants (68–71) but it remains unclear if this same process occurs in mammals.

A number of genes that enhance memory and regulate neuronal migration (e.g., reelin; *Reln*) have now been reported to undergo DNA demethylation and are likely do so to support dynamic mechanisms in the brain, such as synaptic plasticity (37, 42, 49–51, 54–56). Therefore, DNA methylation changes could regulate and be regulated by behavioral changes and synaptic plasticity to potentiate long-term memory processes (48). Recent evidence suggests that hydroxylation of the methyl group can lead to 5-hydroxymethylation (5hmC), a potentially necessary step for active DNA demethylation (17, 72). Proteins of the Ten-Eleven-Translocation (TET) family can catalyze the hydroxylation of 5-methylcytosine (5mC).

Recent reports suggest that TET enzymes participate in the formation of 5-formylcytosine and 5-carboxylcytosine through iterative oxidation of 5mC and 5hmC. Although unknown decarboxylase and base excision mechanisms have been proposed to mediate the replacement of the oxidized base, the precise mechanisms and number of pathways involved remain elusive (73, 74). Interestingly, 5hmC is present in neurons, with brain tissue reported to have the highest levels in the body (75, 76). 5hmC might be an intermediate that recruits several mechanisms, including glycosylation and deaminiation to produce an unmethylated cytosine (17). In the adult brain, TET1 is promoted by the activation induced deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide (AID/APOBEC) deamination pathway and is sufficient to demethylate 5hmC. This pathway can generate a 5-hydroxymethyluracil (5hmU) from a 5hmC, creating a mismatch that will be repaired by the base excision repair (BER) pathway (55). DNA demethylation of *Bdnf* and *Fgf1b* by the TET1/AID mechanism has been demonstrated in the central nervous system (CNS). Furthermore, the deletion of growth arrest and DNA damage-inducible protein 45 B (GADD45B), a protein that is part of the machinery for BER and nucleotide excision repair, abolishes DNA demethylation of those genes (54, 77). Thus, *Gadd45b,* which is activated during learning and memory in the hippocampus, might be supporting memory by promoting demethylation (77). Proteins implicated in DNA demethylation processes (i.e., TET1, GADD45B, AID) could have different functions throughout the life of an organism, mediating the widespread erasure of 5mC during early embryonic development, while serving a gene specific function in the adult brain (55, 78). It would be of interest to determine the order and kinetics of DNA demethylation events induced by neuronal activity in addition to what signals the specificity of genomic location to the associated enzymes.

Adult neurogenesis has been identified in two brain regions, the subventricular and subgranular zones of the dentate gyrus (79, 80). Interestingly, these brain regions are part of the hippocampus, an integration center of information and memory consolidation. Furthermore, several players involved in neurogenesis are regulated by DNA methylation processes (81). Therefore, there might also be an indirect link through which DNA methylation also supports memory. By facilitating neurogenesis and the dendritic growth of newborn neurons, the hippocampus' capacity for memory formation could be increased (82–84). The transcriptional regulation of *Fgf2* and *Bdnf* is critical for neurogenesis and has been associated with the DNA MBD proteins, MBD1 and MeCP2, respectively. These MBDs participate in the transcriptional regulation of *Fgf2* and *Bdnf* in an activity dependent fashion (39, 85, 86). Furthermore, loss of MeCP2 results in deficits in the maturation of newborn neurons and neuronal differentiation in addition to reduced dendritic spine density (87). Interestingly, non-promoter methylation mediated by DNMT3a also facilitates gene transcription and is required for neurogenesis (24). Thus, neurogenesis and memory formation might require DNA methylation and demethylation events to occur at distinct genomic locations, determined by methyl binding proteins and higher order chromatin organization.

A self reinforcing link between epigenetic changes for memory formation

The dynamic DNA methylation changes that have been observed in the adult brain suggest a high level of epigenetic plasticity. This could be achieved through interplay between DNA methylation and other epigenetic events, such as histone modifications. The investigation of epigenetic changes during stem cell differentiation and tumor progression suggests that DNA methylation and histone acetylation are dynamically linked and occur to maintain a specific state of activity. DNA methylation can be the primary mark for gene silencing, with loss of acetylation being secondary. In contrast, transcriptional repression can be achieved through the loss of acetylation, followed by DNA methylation (88–90). As described above, the DNMT and MBD proteins are required for learning and memory. Importantly, other chromatin modifiers are also critical for long-term memory (91–93), suggesting that an epigenetic interplay might also occur during learning and memory. This crosstalk would add another layer of regulatory control to the process of determining gene specific regulation for the brain's complex plasticity needs.

Recent research suggest that both DNA methylation and histone modifications are important during learning and memory (46, 52, 92, 94, 95). The DNMT inhibitor (DNMTi), 5-azaC, blocks training induced acetylation of histone H3 and memory consolidation, suggesting that DNA methylation is connected to histone acetylation in some way (52, 96). Conversely, intra-hippocampal infusion of a HDAC inhibitor (HDACi) prior to the addition of a DNMTi rescues the memory deficit triggered by DNMTi. This further indicates that crosstalk between DNA methylation and histone acetylation is occurring to regulate memory associated genes (52). This transcriptional state maintained by epigenetic modifications is likely to be dynamic, involving feedback between active (e.g., histone acetylation) and repressive (e.g., DNA methylation) marks.

The rescue of a DNMTi-induced memory deficit by HDACi suggests that DNA methylation might occur as a primary event to potentiate histone acetylation during memory consolidation (52). Interestingly, the protein phosphatase1-histone deacetylase1 (PP1-HDAC1) complex represses cyclic adenosine mono-phosphate (cAMP) response element-binding (CREB), a mediator of histone acetylation, to alter the acetylation status of downstream targets (51, 97). In this context, DNA methylation might indirectly participate in the regulation of histone acetylation by mediating the expression of *Pp1*. Prior to synaptic activity, enhancer genes could be bound by the repressive PP1-HDAC1 complex, preventing their transcription and inhibiting histone acetylation. This status could then be modified by the synaptic activity that triggers silencing of *Pp1*, disrupting the PP1-HDAC1 complex. The result would be enhanced transcription of memory enhancer genes through the binding of CREB and subsequent histone acetylation. Histone acetylation might also exert a primary influence on DNA methylation, as suggested by the ability of HDACi to occlude the memory disrupting effects of DNMTi (98, 99). Once a memory has been acquired and

consolidated, feedback mechanisms are needed to return the system to a homeostatic state where it is primed for future learning events. While the idea has yet to be explored, this might involve transcriptional repression of memory enhancers through an enrichment of CpG methylation and a decrease in histone acetylation (51, 100). Interestingly, recent reports in stem cells suggest that in addition to a mediator of demethylation TET1 also recruits epigenetic repressive complexes associated with histone deacetylation and histone methyl marks (101–104). This process might apply to hippocampal neurons where specific chromatin organization would enable TET1 and other epigenetic modifiers to slide along promoter regions to establish either an active or repressive state.

The cross influence of histone acetylation and DNA methylation has also been observed at the level of epigenetic modifiers associated with these marks. In triggering a behavioral response, neurotrophic factors are regulated by the dissociation of a repressive complex formed by MeCP2 and the histone deacetylase HDAC1 (37, 105). Interestingly, environmental factors and behavior also use epigenetic interplay to regulate the glucocorticoid receptor. Hypomethylation of the promoter leads to an increase in acetylation of histone H3 at lysine 9 (H3K9) (42, 106). Thus, the communication between different types of epigenetic modifications participates in the regulation of specific genes that underlie a complex regulatory network to stably or transiently affect neural function.

Stable DNA methylation in the adult brain as a mechanism to maintain behavioral memories

Some newly acquired memories have been demonstrated to originate in the hippocampus. Over time, the memories continue to consolidate as they are incorporated into their final storage site in the cortex (107). The brain likely requires both dynamic and long lasting molecular changes to support these different stages of memory. The transient and dynamic properties of epigenetic modifications are analogous to the molecular requirement for memory formation. Conversely, the ability of epigenetic modifications to self perpetuate in the absence of the initial triggering stimulus mirrors the need of long-term memory storage. The latter is strikingly similar to the cell-autonomous process of cellular memory, allowing the transmission of non-genetic information through cellular division. DNA methylation represents an interesting candidate mechanism for the stable maintenance of the transitory neuronal events initiated at the time memories are formed (Figure 2) (108).

The intrinsic nature of the dynamic methylation observed in the hippocampus is unlikely to serve as a long-term storage mechanism. Recent work suggests that although hippocampal methylation can be transient, methylation of certain cortical genes is a gradual and stable event that could serve as a molecular storage mechanism (50, 51). This study found that the immediate early gene early growth response 1/zinc finger protein (*Egr1/Zif268*) is demethylated in the dorsomedial prefrontal cortex (prelimbic and anterior cingulate cortices) within an hour of contextual fear conditioning. Interestingly, this demethylation occurred in animals that learned in addition to those that were simply exposed to the novel context. Furthermore, this demethylation persisted for at least 30 days (the longest time point examined), suggesting that the change in DNA methylation status reflects a response to any type of new experience. On the other hand, hypermethylation of the memory enhancer gene, *Reln* and the protein phosphatase calcineurin gene, *Ppp3ca*, thought to be a negative regulator inhibiting memory storage was specific to animals that learned the fear association and persisted as the cortical memory trace was established (50, 109, 110). Calcineurins cortical hypermethylation also persisted for at least 30 days and was associated with increased levels of the gene's transcript upon retrieval. The cortical DNA methylation events appear to be

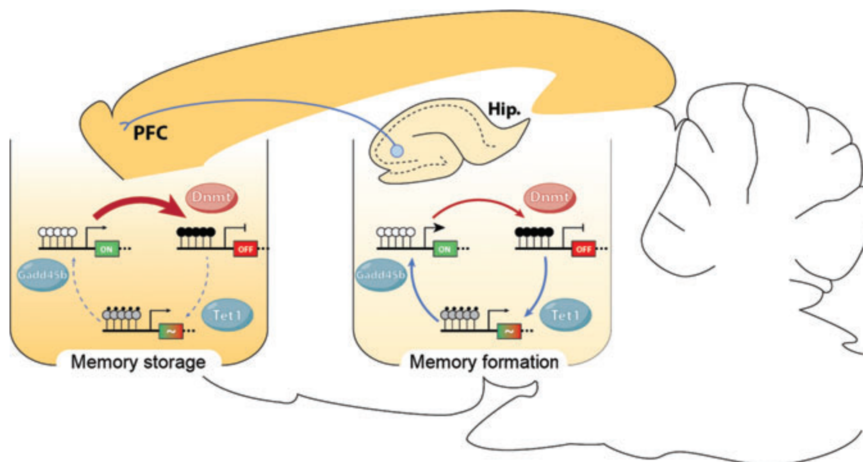

Figure 2 DNA methylation dynamics for memory formation and storage.
Basal DNMT activity might be greater or directed to a specific subset of genes in the prefrontal cortex (PFC) to achieve a stable epigenetic landscape required for memory storage. In the hippocampus, DNA methylation might be more dynamic with additional epigenetic players active to establish a chromatin state targeted by DNMTs and DNA 'demethylases', leading to gene silencing and gene activation.

critical for stable associative memory because intra-cortical inhibition of DNMT1 disrupted the 30 day old fear memory. Interestingly, the establishment of this stable memory trace relies on a dialog between the hippocampus and the cortex, as blockade of hippocampal NMDARs at the time of training prevented learning and cortical hypermethylation of reelin and calcineurin (50). The cortical changes observed at these candidate gene promoters likely apply to a whole host of genes in order to establish a specific transcriptional threshold necessary for intra- and interneuronal synapto-nuclear communication. In this way, a nuclear change could achieve the synaptic specificity required to enable neurons to support multiple memories.

Concluding remarks

Memory acquisition, consolidation and long-term storage recruit DNA methylation in a context specific manner with different kinetics. DNA methylation has primarily been considered a transcriptional regulator that mediates gene silencing. However, its regulation and function are likely to be far more complex in the adult brain and several questions are already apparent. In addition to the proteins that methylate DNA, other epigenetic players are involved and interact with each other (e.g., histone modifications and chromatin remodeling). Recent evidence indicates that non-CpG DNA methylation also has a regulatory function (111). Moving forward, it will be important to go beyond the assessment of methylation patterns at individual gene targets and specific histone modifications to establish the entire epigenetic landscape associated with a specific memory.

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