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DNA methylation and memory formation

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Memory formation and storage require long-lasting changes in memory-related neuronal circuits. Recent evidence indicates that DNA methylation may serve as a contributing mechanism in memory formation and storage. These emerging findings suggest a role for an epigenetic mechanism in learning and long-term memory maintenance and raise apparent conundrums and questions. For example, it is unclear how DNA methylation might be reversed during the formation of a memory, how changes in DNA methylation alter neuronal function to promote memory formation, and how DNA methylation patterns differ between neuronal structures to enable both consolidation and storage of memories. Here we evaluate the existing evidence supporting a role for DNA methylation in memory, discuss how DNA methylation may affect genetic and neuronal function to contribute to behavior, propose several future directions for the emerging subfield of neuroepigenetics, and begin to address some of the broader implications of this work.

The power of self-perpetuation

Experience-dependent behavioral memories can last a lifetime, whereas even a long-lived protein or mRNA molecule has a half-life of around 24 h (ref. 1). Thus, the constituent molecules that subserve the maintenance of a memory will have completely turned over, that is, have been broken down and resynthesized, over the course of about 1 week. However, memories can persist for years or decades. This fact implies the need for self-perpetuating biochemical reactions as a *sine qua non* of long-term memory. These reactions, which are referred to as mnemogenic (memory forming) reactions, have a particular character; one molecule (X), after it is altered or activated as a result of experience (converted to X*), must be able to directly or indirectly catalyze conversion of another molecule of itself (autoconvert) from a nascent into an active form. This peculiar type of biochemical reaction must, of necessity, underlie the molecular perpetuation of memory, as has been discussed previously[2–6]. The memory biochemist must therefore be on the lookout for chemical reactions of this category as candidate mechanisms to potentially underlie the perpetuation of memory. This is what drove the initial interest in the possibility that epigenetic molecular mechanisms, in particular DNA methylation, might sustain memory maintenance.

The self-perpetuating capacity of epigenetic mechanisms in general is nicely illustrated by the process of DNA methylation. DNA methylation is an epigenetic modification in which a methyl group is added to the 5′ position on the cytosine pyrimidine ring[7,8] (**Fig. 1**). This reaction is initiated by *de novo* DNA methyltransferases, yielding the chemical reaction cytosine + DNMT → MeC (methylated cytosine; S-adenosyl methionine is the methyl donor for this reaction). Following this initial methylation step, the MeC then directs methylation on the complementary strand under the control of maintenance DNMTs[8]. The resulting covalent carbon-carbon bond between the carbon atom at the 5′ position on the cytosine ring and the carbon atom in the methyl group is extremely stable, requiring a prohibitively high degree of energy to be directly demethylated[9]. Moreover, on rare occasions when spontaneous demethylation occurs, the complementary strand directs resynthesis of the MeC. Even with oxidative damage to the rest of the cytosine nucleoside, this mechanism allows for regeneration of the MeC, as base excision repair (BER) replaces the defective oxidized nucleoside on one strand and MeC directs its reconversion to MeC[10]. This powerful reaction allows lifelong marking of specific bases in the genome. On this basis, DNA methylation has been referred to as the prima donna of epigenetics[11]. Indeed, this is the mechanism that has been proposed to subserve lifelong maintenance of cellular phenotype (through gene inactivation) after cell fate determination.

Examining a role for DNA methylation in memory formation

With this in mind, neuroscientists began to investigate the possibility that DNA methylation might underlie behavioral memory in the adult CNS. Early studies examined the capacity of behavioral learning in the adult to trigger changes in DNA methylation[12,13]. These studies focused on the hippocampus because it is a brain subregion that is known to be necessary for the establishment of long-term spatial and episodic memory[14,15]. Several pieces of evidence are now available that support the idea that DNA methylation is involved in memory function in the adult CNS. It was previously shown that general inhibitors of DNMT activity alter DNA methylation in the adult brain and alter the DNA methylation status of the plasticity-promoting genes *reelin* and *brain-derived neurotrophic factor* (*Bdnf*)[16]. Additional studies found that *de novo* DNMT expression is upregulated in the adult rat hippocampus after contextual fear conditioning and that blocking DNMT activity blocked contextual fear conditioning[13,17–19]. In addition, fear conditioning is associated with rapid methylation and transcriptional silencing of the memory-suppressor gene *protein phosphatase 1* (*PP1*) and demethylation and transcriptional activation of the plasticity gene *reelin*. These findings suggest that both active DNA methylation and demethylation might be involved in long-term memory consolidation in the adult CNS. A recent series of studies found that the *Bdnf* gene locus is also subject to memory-associated changes in DNA methylation and that this effect is regulated by the NMDA receptor[12], and that neuronal DNMT-deficient animals have deficits in contextual fear conditioning,

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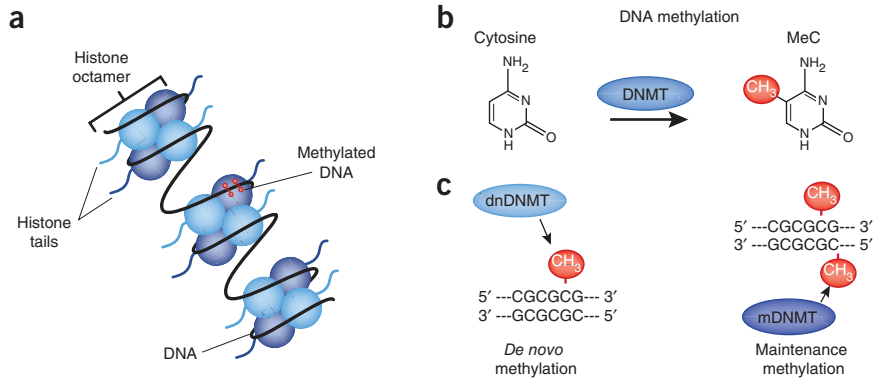

Figure 1 DNA methylation. (**a**) Inside a cell nucleus, DNA is wrapped tightly around an octamer of highly basic histone proteins to form chromatin. Epigenetic modifications can occur at histone tails or directly at DNA via DNA methylation. (**b**) DNA methylation occurs at cytosine bases when a methyl group is added at the 5′ position on the pyrimidine ring by a DNMT. (**c**) Two types of DNMTs initiate DNA methylation. *De novo* DNMTs methylate previously nonmethylated cytosines, whereas maintenance DNMTs methylate hemi-methylated DNA at the complementary strand.

Morris maze and hippocampal long-term potentiation[17]. Overall, these various results suggest that DNA methylation is dynamically regulated in the adult CNS in response to experience and that this cellular mechanism is a crucial step in memory formation. It should be noted that these findings suggest that memory formation involves both increased methylation at memory suppressor genes and decreased methylation at memory promoting genes. Thus, memory function might be driven by either hypermethylation or hypomethylation. Overall, these observations suggest that DNMT activity is necessary for memory and that DNA methylation may work in concert with histone modifications, which have previously been implicated in memory formation and storage in the adult rat hippocampus and cortex[18,20–24].

However, three unanticipated observations arose as part of these studies as well. First, the changes in hippocampal DNA methylation reversed and returned to control levels within 24 h of training. Thus, the duration of this reaction is hardly compatible with the long-lasting mnemogenic reaction discussed above. Second, memory was also associated with demethylation of DNA at some gene loci, which was unexpected because of the chemical strength of the MeC DNA modification. Third, the nucleoside analog DNMT inhibitors that block memory formation (zebularine and 5-aza-2′-deoxycytidine) triggered DNA demethylation as expected, but these agents require chemical incorporation into DNA to be effective. This would normally occur as part of DNA replication in dividing cells. However, the vast majority of cells in the mature CNS do not divide. How then could these agents work? These three considerations indicate that there must be a DNA demethylating activity for the observations to be true. This was not a trivial consideration; even the existence of a DNA demethylase has been controversial[25–27], despite several recent reports that DNA methylation status can cycle at relatively short time scales[28,29]. Currently, the molecular basis of this mysterious demethylating capacity is unclear.

The mysterious demethylating mechanism

Given that the MeC chemical bond is extremely stable, direct demethylation is highly unlikely. An alternative model for DNA demethylation was recently proposed[10,30] (**Fig. 2**). This model involves the conversion of MeC to thymine through deamination or loss of the amine group. Next, following conventional BER, a nonmethylated cytosine is re-synthesized. The precise mechanisms underlying this catalysis are controversial[31,32]. However, it is thought that the growth arrest and DNA damage–inducible protein 45 (GADD45) family of proteins (specifically GADD45β) could participate in each step of this process, thereby catalyzing DNA demethylation[10,30]. Moreover, it appears that DNMTs may be involved in deamination of MeC in a strand-specific manner[29], which would implicate them in both the methylation and demethylation of DNA.

Although it remains unclear whether this model could account for demethylation of both DNA strands, this mechanism would enable selective demethylation at specific sites in DNA, allowing transience of methylation, active demethylation and a route of entry for the nucleoside analog inhibitors of DNMTs into the DNA of nondividing cells. Specifically, after becoming phosphorylated by cytidine kinases, prodrugs such as 5-aza-2′-deoxycytidine or zebularine may operate by substituting for cytosine during BER. This altered base is resistant to methylation and traps DNMTs[33], resulting in both the demethylation of the newly repaired strand and a decrease in DNMT activity. This provides a satisfying explanation for the results described above: a mechanism for reversal of DNA demethylation, a mechanism for active demethylation in nondividing cells and a molecular basis for nucleoside DNMT inhibitors to act in the mature CNS.

Refutation of the initial hypothesis

The discovery of the transience of DNA methylation via these DNA demethylating and remethylating processes negates the broad initial hypothesis that motivated the studies. This initial hypothesis was that the self-perpetuating methylation reaction would underlie memory maintenance. However, these studies actually demonstrated plasticity of DNA methylation in the mature CNS, suggesting that there were previously unknown mechanisms, such as experience-dependent DNA demethylation, and that chemical modification of DNA was involved in memory formation. However, these results refute the idea that these mechanisms act as a long-term molecular storage device, suggesting that DNA demethylation is a much more dynamic process than previously thought (at least in the hippocampus).

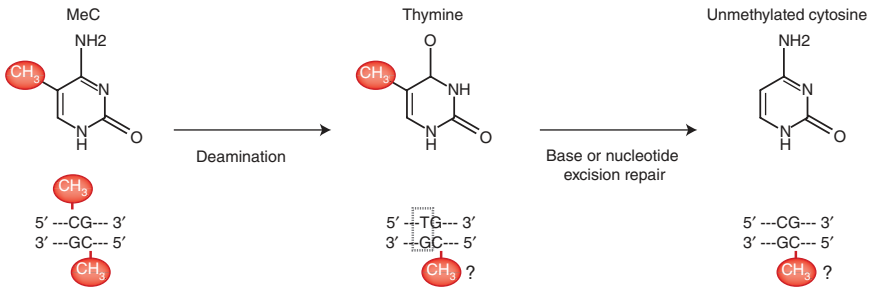

Figure 2 Potential mechanism for demethylation of methylated DNA. Methylated DNA is deaminated and converted to thymine. Base or nucleotide excision repair processes are then able to replace thymine with unmethylated cytosine. It is unclear how this potential mechanism would affect methylation status on the complementary DNA strand.

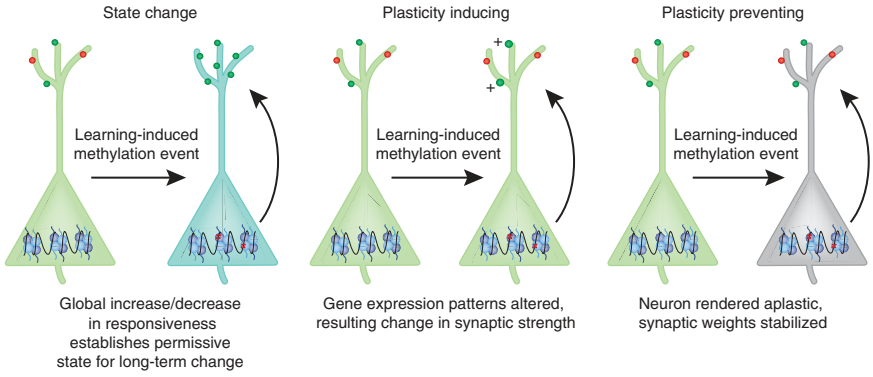

Figure 3 Putative actions of cell-wide DNA methylation changes on neuronal function. Changes in DNA methylation could induce a state change (left) that alters responsivity to existing inputs and acts permissively to enable other long-term changes that are ultimately responsible for memory. Altered patterns of DNA methylation could also directly or indirectly alter gene expression and contribute to changes in synaptic strength that are thought to underlie the formation and maintenance of memories (center). Alternatively, changes in methylation status in a cell may act to render it aplastic, in effect stabilizing the current synaptic weights and responsivity (right). Critically, these changes may occur in different brain regions or at different time points as part of the overall process of learning, memory consolidation and memory maintenance. It is important to note that the changes in DNA methylation driving altered neuronal function are likely to occur at a small subset of the total methylation sites in the cell so that the overall neuronal phenotype is preserved. It also is worth considering that because the methyl-DNA binding proteins do not effectively recognize hemi-methylated DNA, hemi-demethylation of DNA is likely to be just as effective as double-stranded demethylation at triggering functional changes in the neuron.

However, these early studies all focused on the hippocampus, hippocampal synaptic plasticity and hippocampal neuron function[13,16,18]. Although the hippocampus is critical for memory consolidation, it is not essential for long-term memory storage. Thus, the observations of plasticity of DNA methylation in the hippocampus are consistent with the behavioral and systems role of this neuronal circuit and brain subregion. For these reasons, new studies have turned their attention to the cortex, which is a site of long-term memory storage[19,34–36].

It has recently been shown that contextual fear conditioning can induce robust, long-lasting changes in DNA methylation in the anterior cingulate cortex[19]. In fact, such changes were found to last at least 30 d following conditioning, the longest time point that was investigated. Moreover, remote (very long lasting) memory for contextual fear conditioning can be reversed by infusion of DNMT inhibitors into the anterior cingulate cortex, indicating that ongoing perpetuation of DNA methylation occurs in the cortex and is necessary for stabilizing memory. Taken together, these observations are highly consistent with the hypothesis of self-perpetuating methylation and suggest an ongoing need for methylation maintenance and the existence of a true $X + X^* \rightarrow X^* + X^*$ reaction in this brain region for the maintenance of memory.

How does the persisting change in methylation get translated into a functional memory-subserving change in the cortex? This question is especially important given that the readout of DNA methylation is presumed to be cell wide, whereas current models of memory maintenance emphasize synapse-specific changes in function. In terms of how the epigenetic marks are transformed into functional consequences in the cell, there are three broad possibilities (**Fig. 3**). First, DNA methylation changes may drive a change in the response state of the neuron that is permissive for other mechanisms to establish and maintain more permanent changes. Second, methylation events may actively participate in altered the gene readout that contributes to ongoing memory; for example, by enhancing synaptic strength. Third, the most unusual concept is that epigenetic mechanisms might actually render the cell totally aplastic, stabilizing a given distribution of synaptic weights as a necessary condition for memory stability. Layered on all three possibilities is the conundrum of how cell-wide changes (driven by epigenetic marks) can be participating in the face of the apparent necessity of synapse specificity in memory circuits. The last mechanism addresses this in a simple fashion, which is an appealing aspect of this idea. It is worth noting that the first two ideas are not mutually exclusive, even in the same cell. In terms of the entire memory storage circuit, all three mechanisms could possibly be involved at different sites or at different times. As epigenetic changes occur downstream of synaptic activity, they have the ability to integrate multiple cellular signals and modulate the long-term responsiveness of a neuron by controlling gene expression. In terms of memory storage, epigenetic changes may therefore enable cells to effectively cement a specific response to a given set of inputs by controlling the degree of plasticity that occurs at all synapses. In this manner, memory storage may be conceptually thought of as both a synaptic process that controls the nature of signals that a cell receives and an epigenetic process that controls subsequent expression of memory-related genes.

Neuroepigenetics in the context of traditional epigenetics

One of the traditional definitions of epigenetic mechanisms requires that for something to be 'epigenetic' it must be heritable, either across the germ line or across cell division[37]. Obviously, as neurons cannot divide and are not germ cells, nothing that occurs in neurons in the adult CNS would qualify as being epigenetic by this definition. However, a wide variety of data indicate that active regulation of chromatin structure and DNA methylation are critical for the ongoing function of the mature CNS. In a broad sense, these processes might be described as being neuroepigenetic to distinguish them from heritable epigenetic marks involved in development, cell-fate determination and cell division. For this reason, we use the term neuroepigenetic to describe the concept that cells in the mature CNS may have specialized adaptations of the epigenetic biochemical machinery to provide regulatory processes that may not be widely used in other cell types (see, also ref. 38). We define neuroepigenetics as a potential subfield of epigenetics that deals with the unique mechanisms and processes allowing dynamic experience-dependent regulation of the epigenome in nondividing cells of the nervous system, along with the traditionally described developmental epigenetic processes involved in neuronal differentiation and cell-fate determination.

We speculate that the new understanding of the role of neuroepigenetic molecular mechanisms in memory formation can answer the long-standing question in neuroscience of why neurons can't divide. The fact that neurons have co-opted epigenetic mechanisms to subserve long-term functional changes may preclude their use of these same mechanisms to perpetuate cellular phenotype with cell division. In a sense, the neuron can't have its cake and eat it too; it can either use epigenetic molecular mechanisms to perpetuate cell

fate across cell division or use a subset of them to perpetuate acquired functional changes across time, but not both. Obviously, this remains our speculation and future investigations will be required to fully address this hypothesis. Notably, accumulating evidence indicates that DNA methylation is also involved in the development, survival and function of newborn neurons in the subventricular and subgranular zones of adult animals[30,39,40], revealing yet another potential locus for neuroepigenetic mechanisms to influence the function of the mature CNS. Nevertheless, it remains unclear whether the epigenetic modifications that underlie conversion of neural stem cells into mature adult neurons overlap with the mechanisms responsible for long-term maintenance of functional change.

Relationship to systems neuroscience

The idea that epigenetic modifications regulate the formation, maintenance and expression of memories does not diminish the importance of circuit-level phenomena in learning and memory. In fact, to understand how DNA methylation could contribute to memory, it is first necessary to understand how neural circuits encode, consolidate and store memory-related information. For example, contextual fear conditioning produces transient changes in DNA methylation in the hippocampus, but prolonged changes in DNA methylation in the cortex. Our speculation is that there are actually two different mechanisms, one that participates in consolidation (hippocampus) and one that participates in storage (cortex). Together, these mechanisms could allow for plasticity in hippocampal circuits to enable rapid consolidation and stability in cortical circuits to promote the long-term maintenance of memory. As the hippocampus is needed to form new, subsequent memories, its epigenetic mechanisms may have to be plastic to allow the system to reset after it has served its function.

We speculate that the manner in which a brain region uses epigenetic modifications to regulate memory will differ on the basis of the functional roles of that structure. Indeed, unique properties for the regulation of DNA methylation may be conferred by regional differences in the kinetics or expression of DNA methylation modifying enzymes, as have recently been discovered in subregions of the hippocampus[41]. *Vis-à-vis* the epigenetic heritability issue raised above, there may be an interesting analogy in this. DNA marks generated in the hippocampus may be 'heritable' in the CNS in the sense that the hippocampal circuit, driven by altered DNA methylation, downloads epigenetic marks from the hippocampus to the cortex. The specific marks would not be the same in hippocampus and cortex, but, in a broad sense, transient methylation marks in the hippocampus would drive the establishment of persisting methylation marks in the cortex. We could call this 'systems heritability' of epigenetic marks.

Upstream regulation and readout mechanisms

To promote memory formation, changes in DNA methylation must be selective, potentially even at the single-nucleotide level. The neuron cannot risk dedifferentiation; thus, plastic sites must be compartmentalized from maintenance sites and sites involved in the perpetuation of cellular phenotype. At present, the upstream mechanisms that regulate this process are very mysterious and it is unclear how one specific site or gene region is targeted for methylation or demethylation in any cell type[42]. However, recent discoveries have pointed toward neuron-specific mechanisms. For example, hydroxymethylcytosine (OH-MeC) has been found at high levels in neural tissue[43,44]. Although the function of OH-MeC is not known, it is noteworthy that it possesses a lower affinity for proteins with methyl-binding domains, such as MeCP2, than does MeC[45]. Thus, it is possible that OH-MeC could be a chemical precursor to target sites for active demethylation or may even constitute a plastic mechanism for reversibly negating the effects of methylation.

How might selective modifications of specific C and G dinucleotides in an entire genome be attained? Recent findings indicate that one component of specificity in altering DNA methylation profiles may be conferred via histone modifications that encourage the binding of DNMTs to DNA. For example, the *de novo* methyltransferase DNMT3a binds to DNA with a greater efficiency when lysine 9 on H3 is trimethylated than when lysine 4 on H3 is trimethylated[46]. Conversely, entire stretches of nonmethylated CpGs may be preserved despite global DNMT activity by proteins such as Cfp1, which binds selectively to nonmethylated CpG islands and may assist in the perpetuation of this state via interactions with H3K4 methylation[47]. Thus, DNA methylation may be specifically guided by some chromatin modifications and permanently inhibited by others, resulting in a multi-layered regulation of methylation patterns.

Changes in DNA methylation may therefore affect neuronal activity in many ways, most of which are only beginning to be understood. Although DNA methylation was once mainly associated with transcriptional repression, it is also possible that DNA methylation can result in transcriptional activation in the CNS[48,49]. Given this, a final consideration is which gene products may be targeted for epigenetic modification that in turn result in changes in synaptic strength or the capacity for synaptic plasticity? The answer to this question is unknown. However, alterations in DNA methylation or in the proteins that bind to methylated DNA produce robust changes in the expression patterns of several genes that have been implicated in synaptic plasticity, including *Bdnf*, *calcineurin*, *PP1* and *reelin*[12,19,30,34,50]. Similarly, inhibition of DNA methylation disrupts long-term potentiation in the hippocampus, providing additional evidence for its involvement in neuronal plasticity[16]. Thus, DNA methylation could potentially have multiple roles in neuronal change, all of which may also be regionally, temporally and neuronally specific. In fact, understanding how epigenetic mechanisms contribute to functional change in diverse neuronal populations is an especially important issue that will come with its own challenges. As unique sets of cells perform specific functions in a neuronal circuit, and each cell in this set maintains its own epigenome, discovering which epigenetic mechanisms are used by specific neuronal phenotypes will be critical for relating epigenetic changes to neuronal function. Adding to this difficulty is the fact that discrete neuronal populations often physically overlap in the same brain region, making it harder to assay the epigenetic status of any given neuronal phenotype.

It is clear that we have not yet begun to determine in a comprehensive fashion how DNA methylation at the cellular level gets translated into altered circuit and behavioral function. Thus far, most studies have been restricted to using a candidate target gene approach to identify specific sites of methylation changes. However, these data only allow for the assessment of a small subset of changes in DNA methylation. It is not yet possible to try to mechanistically tie these specific changes at single gene exons to complex multicellular, multicomponent processes, such as long-term potentiation, hippocampal circuit stabilization and behavioral memory, as the molecular approaches are limited to sampling such a small subset of genes. Thus, a future challenge for neuroepigenetics researchers will be to expand the level of analysis using sophisticated epigenome-wide screens[17], potentially revealing a myriad of functional effector genes subjected to epigenetic control and perhaps identify previously unknown mnemogenic molecules.

In summary, all of these considerations imply the existence in neurons of specialized epigenetic biochemical machinery and processes that may not exist in other cell types. Regardless of the nomenclature,

future studies will hopefully yield an increased understanding of the processes subserving the epigenetic code operating in memory formation, as well as other long-lasting forms of behavioral change.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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