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DNA methylation and Memory Formation

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Introduction

Memory formation and storage require long-lasting changes within memory-related neuronal circuits. Recent evidence indicates that DNA methylation may serve as a contributing mechanism in memory formation and storage. These emerging findings both 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. This perspective will 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 hrs¹. Thus, the constituent molecules that subserve the maintenance of a memory will have completely turned over, i.e. have been broken down and resynthesized, over the course of about 1 week. Yet 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 (C) pyrimidine ring^{7, 8} (see Figure 1). This reaction is initiated by *de novo* DNA methyltransferases (dnDNMTs), yielding the chemical reaction $C + DNMT \rightarrow MeC$ (S-adenosyl methionine is the methyl donor for this

reaction). Following this initial methylation step, the methylated cytosine then directs methylation on the complementary strand under the control of *maintenance DNMTs* (mDNMTs)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 demethylated⁹. 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 regeneration of the MeC, as Base Excision Repair (BER) replaces the defective oxidized nucleoside on one strand and MeC directs its reconversion to MeC¹⁰. This powerful reaction allows lifelong marking of specific bases within the genome. On this basis, DNA methylation has been referred to as the *prima donna* of epigenetics¹¹. Indeed, this is the mechanism 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. Some of the first studies looked at the capacity of behavioral learning in the adult to trigger changes in DNA methylation^{12, 13}. These first studies focused on the hippocampus because it is a brain subregion 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 plays a role in memory function in the adult CNS. Work by Levenson et al.¹⁶ demonstrated 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)*. Additional studies demonstrated that *de novo* DNMT expression is up-regulated 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 (PPI)* and demethylation and transcriptional activation of the plasticity gene *reelin*. These findings have the surprising implication 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 receptor¹², and that neuronal DNMT deficient animals have deficits in contextual fear conditioning, Morris maze, and hippocampal LTP¹⁷. 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 is important to note 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 hours after training the animals. Therefore, the duration of this reaction is hardly compatible with the long-lasting mnemogenic reaction discussed above. Secondly, memory was also associated with *demethylation* of DNA at some gene loci, which was unexpected due to 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? All three considerations imply the existence of a *DNA demethylating activity* in order 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. Recently, Song et al¹⁰ have proposed an alternative model for DNA demethylation based on recent exciting results from their laboratory³⁰ (see Figure 2). The model involves the conversion of methylated cytosine to thymine through deamination, or loss of the amine group. Next, following conventional BER, a non-methylated 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 GADD45 β) could participate in each step of this process, thereby catalyzing DNA demethylation^{10, 30}. Moreover, it appears that DNMTs may also play some role in deamination of methylated cytosine in a strand specific manner²⁹, giving them a role in both 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: 1) Transience of methylation, 2) active demethylation, and 3) a route for entry of for the nucleoside analogue inhibitors of DNMTs into the DNA in non-dividing cells. Specifically, after becoming phosphorylated by cytidine kinases, prodrugs like 5-aza-2'-deoxycytidine or zebularine may operate by substituting for cytosine during BER. This altered base is resistant to methylation and also traps DNMTs³³, resulting in the demethylation of the newly repaired strand as well as a decrease in DNMT activity. This provides a satisfying explanation for the unanticipated results described above – a mechanism for reversal of DNA demethylation, a mechanism for active demethylation in non-dividing cells, and a molecular basis for nucleoside DNMT inhibitors to act in the mature CNS.

Refutation of the initial hypothesis

The transience of DNA methylation via these DNA demethylating and remethylating processes also negates the broad initial hypothesis motivating the studies. The initial idea was that the self-perpetuating methylation reaction would underlie memory maintenance. However, the initial studies actually demonstrated *plasticity* of DNA methylation in the mature CNS, implying novel mechanisms like experience-dependent DNA demethylation and a role for chemical modification of DNA in memory formation. However, they refuted the potential role of these mechanisms as a long-term molecular storage device, thus revealing that DNA de/methylation is much more dynamic process than previously thought (at least in the hippocampus).

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}.

Recent observations have shown that contextual fear conditioning can induce robust, long-lasting changes in DNA methylation in the anterior cingulate cortex (ACC)¹⁹. In fact, such changes were found to last at least 30 days 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 ACC, demonstrating that ongoing perpetuation of DNA methylation occurs in the cortex and is necessary as a memory stabilizing mechanism. 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 since a subtext here is 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 (Figure 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, e.g. 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 a role for synapse specificity in memory circuits. The last mechanism addresses this in a simple fashion, which is an appealing aspect of this novel idea. It is worth noting that the first two ideas are not mutually exclusive, even within the same cell. In terms of the entire memory storage circuit, all three mechanisms could possibly play a role at different sites or at different times. Since 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 way, 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 division³⁷. Obviously, since neurons cannot divide and are not germ cells, by this definition nothing that occurs in neurons in the adult CNS would qualify as “epigenetic”. However, a wide variety of data now demonstrate that active regulation of chromatin structure and DNA methylation are processes critical to the ongoing function of the mature CNS. In a broad sense these processes might be described as neuroepigenetic to distinguish them from heritable epigenetic marks involved in development, cell fate determination, and cell division. For this reason in this article we and others use the term *neuroepigenetic* to try to capture the concept that cells in the mature CNS may have specialized adaptations of the epigenetic biochemical machinery, in order to provide regulatory processes that may not be widely utilized in other cell types (also see 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 non-dividing 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. Interestingly, 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 in play, 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 in order to allow the system to reset after it has served its function. We speculate that how a brain region uses epigenetic modifications to regulate memory will differ based on 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 within subregions of the hippocampus⁴¹. Vis-a-vis the epigenetic heritability issue raised above, there may be an interesting analogy here. DNA marks generated in the hippocampus may be “heritable” within 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 be driving 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, so plastic sites must be compartmentalized from maintenance sites, from 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 type⁴². However, recent discoveries are already suggesting 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 MeC⁴⁵. 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 to reversibly negate the effects of methylation.

How might selective modifications of specific C–G dinucleotides within an entire genome be attained? Recent findings indicate that one component of specificity in altering DNA methylation profiles may be conferred by via histone modifications that encourage the binding of DNMTs to DNA. For example, the de novo methyltransferase DNMT3a binds to DNA with more efficiency when lysine 9 on H3 is trimethylated than when lysine 4 on H3 is trimethylated⁴⁶. Conversely, entire stretches of non-methylated CpGs may be preserved despite global DNMT activity by proteins such as Cfp1, which bind selectively to non-methylated CpG islands and may assist in the perpetuation of this state via interactions with H3K4 methylation⁴⁷. 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 may also result in transcriptional activation in the CNS^{48, 49}. Given this, a final consideration is what the gene products are that 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 essentially completely unknown at present. 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*, *PPI*, and *reelin*^{12, 19, 30, 34, 50}. Likewise, inhibition of DNA methylation disrupts long-term potentiation within the hippocampus, providing additional evidence of its role in neuronal plasticity¹⁶. Thus, DNA methylation could potentially play multiple roles in neuronal change, all of which may also be regionally, temporally, and even 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. Since unique sets of cells perform specific functions within a neuronal circuit, and each cell within 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 within 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 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 like LTP, hippocampal circuit stabilization, and behavioral memory at this point, because of the limitation that the molecular approaches are sampling such a small subset of genes. Thus, a future challenge for neuroepigenetics researchers will be to expand the level of analysis by incorporating sophisticated epigenome-wide screens

into the technical repertoire¹⁷, potentially revealing a myriad of functional effector genes subjected to epigenetic control and perhaps identify novel 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 increasing understanding of the processes subserving the epigenetic code operating in memory formation, as well as other long-lasting forms of behavioral change.

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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 DNA methyltransferase (DNMT). **c**, Two types of DNMTs initiate DNA methylation. De novo DNMTs methylate previously non-methylated cytosines, whereas maintenance DNMTs methylate hemi-methylated DNA at the complementary strand.

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 panel) which alters responsiveness to existing inputs and acts permissively to enable other long-term changes which 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 panel).

Alternatively, changes in methylation status within a cell may act to render it aplastic, in effect stabilizing the current synaptic weights and responsiveness (right panel). 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, in order that the overall neuronal phenotype be 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 just as effective as doublestranded demethylation in triggering functional changes in the neuron.