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DNA methylation

A transcriptional mechanism co-opted by the developed mammalian brain?

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Forming and maintaining behavioral memories is a complex process that involves, among other requirements, transcriptional regulation and systems communication. Here we review recent studies exploring the role of DNA methylation in these critical processes. Further, we suggest that, perhaps, the adult brain controls and utilizes the mechanism of DNA methylation in non-traditional ways that are waiting to be explored.

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The ability to learn and remember is an evolutionarily critical function of the brain. For example, we have all experienced the conserved phenomenon of conditioned taste aversion (CTA). CTA is a form of memory that instructs the brain to avoid any food source that was consumed in close temporal proximity to the development of nausea. Without CTA memories, we would risk death by returning to tainted food sources. Similarly, fear conditioning involves associating danger with something in our environment that was previously innocuous. Future encounters with this environmental stimulus recall memories of the fearful association, enabling a behavioral response directed towards safety. While the fear response varies across species from freeze, fight or flight, the same associative memory is responsible for driving survival behaviors.

CTA and learned fear are examples of relatively simple behaviors. However, the behavioral output is only the proverbial tip of the iceberg. An astounding complexity is operating just beneath the surface to produce the behavior. Processes required for the long-term memories that direct these behaviors include (1) ordered communication between different brain regions to process multiple modes of external input, (2) the orchestration of cellular activation at discrete sites on specific cell types, (3) a tightly regulated program of gene transcription (**Fig. 1**), (4) protein translation and subsequent trafficking to the synaptic site of activation, (5) further ordered communication between different brain regions to direct appropriate motor output at the right times and (6) largely unknown molecular mechanisms that maintain the newly formed synaptic contacts representing the memory trace. Further, every one of these processes has critical temporal and spatial requirements. For instance, if transcription is temporarily blocked an hour after associative training, memory fails. The importance of a countless number of specific genes and signaling pathways for successful memory has been established in recent decades (reviewed in ref. 1).

The implications of needing a tightly regulated program of gene transcription likely shouts "epigenetic regulation" to someone with a background in the field of epigenetics. Indeed, given the complexity of memory, it is not surprising that the brain might co-opt an available mechanism developed to serve other purposes in the body. It certainly would not be the first time (**Fig. 1**).[2,3] In the current case, the hypothesis is that the adult brain makes use of a master regulator of transcription, DNA methylation, to orchestrate the complex transcriptional processes critical for the regulation of memory. Beyond the transcriptional pressure induced by memory's complexity, three factors encouraged us to investigate a potential role for DNA

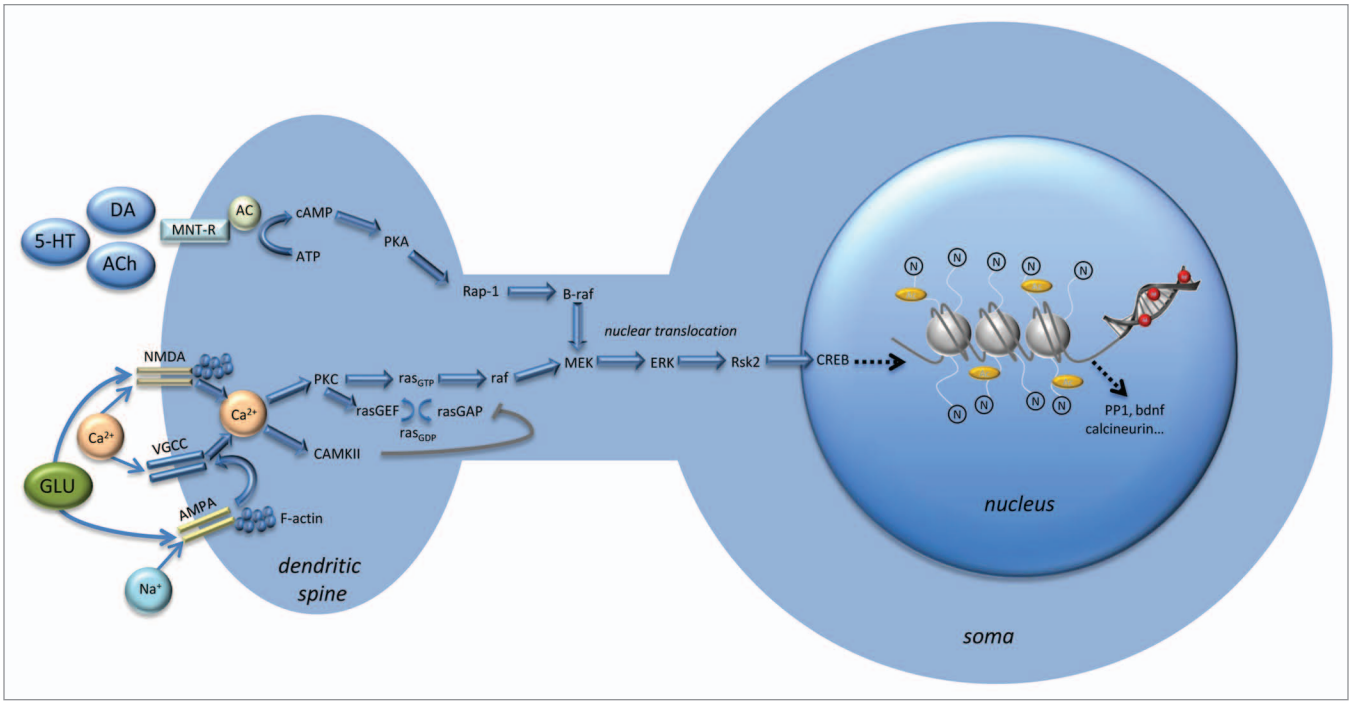

Figure 1. Epigenetic mechanisms are necessary for the tightly regulated transcriptional program supporting memory. This figure is a simplified representation of synaptic and nuclear activities required for memory. The mechanisms responsible for activating epigenetic mechanisms with learning (particularly DNA methylation) are largely unknown.

methylation in memory. First, initial explorations into epigenetic mechanisms and memory focused on histone modifications and the results were extremely encouraging. Briefly, studies focused on histone acetylation established that learning induces acetylation of histone H3, the histone acetyltransferase (HAT) CREB binding protein (CBP) and histone deacetylases HDAC2 and 3 are critical for long-term memory, and inhibition of HDACs can rescue memory in mouse models of memory failure (e.g., Alzheimer disease, aging).[4-12] Second, in 2004 the Meaney lab demonstrated that poor maternal care could induce DNA methylation changes to the glucocorticoid receptor (GR) in rat pups.[13] Importantly, modifications to the GR promoter altered the quality of care female pups provided in adulthood to their own offspring. This finding demonstrated that environmental factors present during postnatal development could influence the epigenetic landscape of the brain, thus effecting future behavior. Third, the Fan lab examined levels of DNA methyltransferases (DNMTs) in the adult brain and found them to be unexpectedly high for a structure filled with post-mitotic neurons.[14] This suggested that DNMTs might serve a function above and beyond maintaining DNA methylation patterns put in place during development and differentiation.

Newly acquired memories undergo a confined period of consolidation, where the memory traces are strengthened for long-term storage and become less susceptible to interruption. This consolidation period occurs in the hippocampus for many memories and coincides with a lasting increase in synaptic strength, a change thought to be a critical part of successful memory formation. This long-lasting enhancement in the signal transmission between neurons is known as long-term potentiation (LTP). The first evidence suggesting a role for DNA methylation in memory came from the in vitro work of Levenson and colleagues, which demonstrated that LTP induction is associated with changes in hippocampal DNA methylation levels.[15] Further, the potentiation was blocked by DNMT inhibition. Nelson and colleagues also showed that DNA methylation is required for the maintenance of homeostatic synaptic plasticity.[16] The next big leap was moving in vivo to demonstrate a requirement for DNA methylation in the behaving animal.[17] We probed the role of methylation in memory formation using a contextual fear conditioning paradigm in which rats learn to associate a mildly aversive foot shock with a novel context during a single training session. Memory performance can later be assessed by measuring how much time the animal spends freezing when returned to the novel context. Contextual fear learning was associated with increased transcription of the de novo DNMTs (3a and 3b) in the hippocampus, as well as changes in DNA methylation.[17] Specifically, methylation levels of the memory-enhancing gene, *reelin*, decreased. Simultaneously, methylation of the memory-suppressing gene, *PP1*, increased. Both genes demonstrated transcription changes that corresponded to the transcriptional repression associated with DNA methylation. Importantly, these learning-induced methylation changes were prevented by DNMT inhibition at the time of training and returned to basal levels with 24 h. Further, intra-hippocampal DNMT inhibition disrupted the formation of memory.[17] This critical

finding was confirmed in two subsequent studies.[18,19] Interestingly, DNMT inhibition also coincides with suppression of the memory-associated increases in H3 acetylation.[18] Further, we found that pretreatment with an HDAC inhibitor ameliorated the deficits in contextual memory and LTP produced by DNMT inhibition, highlighting the importance of the interplay between different epigenetic modifications.[18]

DNA methylation has been implicated in other memory paradigms, such as novel object recognition. This memory test makes use of a rodent's innate preference for novelty. Memory is measured as an index of the amount of time an animal spends exploring a new object when it replaces an object the animal was previously exposed to. The amount of time spent exploring a novel object is correlated with *bdnf* hypomethylation in the hippocampus.[20] Consistent with this result, elevated hippocampal DNMT expression was also linked to the beneficial memory effects of estrogen in novel object recognition.[21] The importance of DNA methylation in memory formation was further supported by experiments utilizing conditional knockout mice lacking both the maintenance methyltransferase, DNMT1, and the de novo methyltransferase, DNMT3a.[22] Mice lacking forebrain expression of these two enzymes performed worse in the Morris water maze, a task where mice must utilize contextual spatial cues to find a hidden platform in a pool of opaque water. These double knockout mice also displayed deficits in hippocampal LTP.[22]

The studies just described demonstrate that DNA methylation plays a critical and dynamic role in regulating the transcriptional program supporting memory formation. They also raise several intriguing questions. For instance, what mechanisms allow for DNA methylation to bidirectionally change so quickly after associative training? And further, what enables methylation to rapidly return to basal levels after memory formation? This indicates the existence of demethylase activity in the brain in some form. Whether this is enzymatic or a more passive process, such as a DNA repair mechanism, is currently unknown.[23,24] Further, how are S-phase drugs, like 5-azadeoxycytidine and zebularine able to effect promoter methylation in the brain, as well as behavior, when the compounds are infused into a sea of post-mitotic neurons? A wealth of data indicates that the effect of these drugs cannot be attributed to cellular damage or non-specific effects. For instance, the key findings have been replicated with RG108, a direct DNMT inhibitor.[17-19] These very real issues can be distilled down to one fundamental question—How does DNA methylation operate in post-mitotic neurons of the adult brain? Much of the data suggests the rules might be different in the mammalian central nervous system (CNS). For decades, neuroscientists have been addressing the question of how our external environment influences our genomic environment and vice versa. This makes us well prepared to investigate the effects of epigenetic modifications on cognition. However, in comparison to epigeneticists, we are ill equipped to study the mechanisms that regulate the operation of DNA methylation itself in post-mitotic neurons (**Fig. 1**). It is our hope that epigeneticists will be intrigued by the unique challenge presented by the brain and illuminate the mechanisms regulating DNA methylation in the CNS.

A persisting question in the field of memory is how the brain maintains memories for months and even years. Because the half-lives of proteins supporting the initial formation of memory are much shorter than a behavioral memory's lifetime, it seems that the brain is in need of some sort of self-perpetuating signal to maintain a memory trace beyond the first few days. In light of DNA methylation's reputation of stability, we measured hippocampal methylation levels 24 h after learning to see if learning-induced methylation changes could contribute to memory maintenance. Unfortunately, methylation returns to baseline in the hippocampus within 24 h of training.[17] This indicates that DNA methylation is regulated in a highly dynamic fashion in the developed hippocampus and is unlikely to participate in memory maintenance. However, this might fit with a time-limited role for the hippocampus in memory. As described above, early memory consolidation mechanisms occur at the synaptic level to support long-term memories. However, a quickly growing body of evidence suggests that memory traces undergo an additional, more prolonged consolidation process at a systems level (on the order of weeks). This system consolidation involves a gradual reorganization of the neural network over time and the reorganization is reflected by a transition of the neural substrates supporting a memory from the plastic hippocampus to the more stable neocortex. Consistent with this, we recently demonstrated that initial hippocampus-dependent fear learning induced DNA methylation changes in the prefrontal cortex (PFC) that were required for memory maintenance.[25] Of particular note is the heightened promoter methylation of *calcineurin*, a memory suppressor, within 24 h of learning. Contrary to DNA methylation events in the hippocampus, this change lasted at least 30 days. Further, administration of DNMT inhibitors into the PFC 30 days after learning disrupted the fear memory.[25] These results demonstrate that DNA methylation serves a more traditional role in the cortex by stably marking a gene promoter as a means of contributing to the long-term maintenance of a memory.

One important question from the perspective of memory and CNS efficiency is why the brain would expend the energy required to shift control over a memory from one brain region (hippocampus) to another (cortex). It appears that this seemingly inefficient process may serve to support the meticulous integration of the components of a new memory into the complex network of memories already present in the cortex. The concept of cortical integration can easily be understood through simple observations of how our own minds work. Recall of a certain memory, such as visiting your grandmother, effortlessly recalls related memories (e.g., what her favorite hat looks like, the smell of cookies baking, the color of your grandfather's favorite chair, pictures hanging on the walls, etc.). Linking memory traces to each other at points of commonality (e.g., all things "grandmother") is a far more efficient method of accessing and utilizing memories than effortful recall of each individual memory associated with your grandmother. The benefit of this high

level of integration appears to outweigh the energy cost of moving a memory trace around the brain. We have established that cortical promoter methylation persists into the late post-training time points when a memory relies on the cortex. This supports the importance of DNA methylation in memory maintainenance. But an additional exciting and unexpected possibility is that DNA methylation also supports the regional movement of memories. DNA methylation changes in the cortex actually occur on a surprisingly early timescale after hippocampus-dependent learning (e.g., within hours of training), indicating that methylation may be one of the earliest mechanisms to participate in the incorporation of a memory trace into the cortical network.

Collectively, these studies provide compelling evidence for the involvement of DNA methylation in learning and memory. These studies also suggest that DNA methylation can provide an organism with a dynamic mode to regulate transcription of genes important to memory function during the earlier period of synaptic consolidation. However, DNA methylation also seems capable of serving as a more stable epigenetic marker during system consolidation. One question lurking on the horizon: What mechanisms are employed by the CNS to regulate these qualitatively different modes of DNA methylation?

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