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Covalent Modification of DNA Regulates Memory Formation

Courtney A. Miller[1] and J. David Sweatt[1,*]
[1] Department of Neurobiology and the Evelyn F. McKnight Brain Institute,University of Alabama at Birmingham, Birmingham, AL 35294, USA
*Correspondence: dsweatt@nrc.uab.edu
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SUMMARY

DNA methylation is a covalent chemical modification of DNA catalyzed by DNA methyltransferases (DNMTs). DNA methylation is associated with transcriptional silencing and has been studied extensively as a lifelong molecular information storage mechanism put in place during development. Here we report that DNMT gene expression is upregulated in the adult rat hippocampus following contextual fear conditioning and that DNMT inhibition blocks memory formation. In addition, fear conditioning is associated with rapid methylation and transcriptional silencing of the memory suppressor gene *PP1* and *de*methylation and transcriptional activation of the synaptic plasticity gene *reelin*, indicating both methyltransferase and demethylase activity during consolidation. DNMT inhibition prevents the *PP1* methylation increase, resulting in aberrant transcription of the gene during the memory-consolidation period. These results demonstrate that DNA methylation is dynamically regulated in the adult nervous system and that this cellular mechanism is a crucial step in memory formation.

INTRODUCTION

The learning and memory field has long recognized the importance of transcriptional regulation during memory formation. However, we have a relatively poor understanding of how this transcriptional regulation occurs. While a great deal of effort has focused on the role of transcription factors in synaptic plasticity and memory, a burgeoning field is discovering evidence implicating epigenetic mechanisms in the transcriptional regulation underlying long-term memory formation.

Epigenetic mechanisms are essential to normal development, as they provide the cellular memory necessary for perpetuating the correct cellular phenotype during mitosis. The mechanisms that accomplish this are a set of posttranslational modifications of DNA and chromatin that alter gene expression patterns. Rapidly accumulating evidence suggests that the nervous system has co-opted these epigenetic mechanisms utilized during development for the generation of long-term behavioral memories in adulthood (Swank and Sweatt, 2001; Guan et al., 2002; Huang et al., 2002; Levenson et al., 2004a; Korzus et al., 2004; Alarcon et al., 2004; Wood et al., 2005; Kumar et al., 2005; Levenson et al., 2006; Chwang et al., 2006).

DNA is tightly packaged into a DNA-protein complex known as chromatin, and highly basic proteins known as histones are the major component. In chromatin's native state, transcription is repressed through tight binding of histones to DNA, preventing the requisite RNA polymerase II/DNA interaction (Varga-Weisz and Becker, 1998). Therefore, in order to initiate transcription, chromatin's tightly compacted structure must be disrupted. Acetylation of the ε-amino group of lysine residues by histone acetyltransferases (HATs) is one way of accomplishing this conformational change. This disrupts the histone-DNA interaction and facilitates binding of transcription factors and RNA polymerase II to DNA, resulting in increased initiation of transcription (Varga-Weisz and Becker, 1998; Turner, 2002; Battaglioli et al., 2002; Lunyak et al., 2002).

Recent evidence indicates that regulation of chromatin structure serves as an important control mechanism in memory-associated transcriptional regulation (Swank and Sweatt, 2001; Guan et al., 2002; Huang et al., 2002; Levenson et al., 2004a, 2006; Korzus et al., 2004; Alarcon et al., 2004; Wood et al., 2005; Kumar et al., 2005; Chwang et al., 2006). For example, several studies have implicated the HAT activity of CREB binding protein (CBP) in both long-term facilitation in *Aplysia* and the formation of long-term memory in rodents (Guan et al., 2002; Korzus et al., 2004; Alarcon et al., 2004; Wood et al., 2005). Our laboratory has recently found that acetylation and phosphorylation of histone H3 are increased in vitro in the hippocampus following activation of NMDA receptors and extracellular signal-regulated kinase (ERK) (Levenson et al., 2004a). In vivo, contextual fear conditioning is accompanied by similar increases in acetylation and phosphorylation of H3 within the hippocampus (Levenson et al., 2004a; Chwang et al., 2006). Moreover, artificially elevating levels of histone acetylation using histone deacetylase (HDAC) inhibitors enhances induction of long-term potentiation in vitro and formation of long-term memory

in vivo (Levenson et al., 2004a). Thus, a model is emerging whereby hippocampus-dependent memory formation is initiated by activation of NMDA receptors, which leads to an influx of calcium, activation of signaling pathways, and altered gene transcription mediated in part by changes in chromatin structure.

In the current study, we explored the potential role of another epigenetic mechanism, cytosine-5′ methylation, in memory formation. Many developmentally important processes utilize this "prima donna" of epigenetics (Scarano et al., 2005; Santos et al., 2005), including gene imprinting, cell differentiation, X chromosome inactivation, and long-term transcriptional regulation (Bestor et al., 1988; Okano et al., 1998). This covalent modification of DNA is catalyzed by DNA (cytosine-5′) methyltransferases (DNMTs) and involves the transfer of a methyl group to the 5′ position of cytosine residues, canonically at CG dinucleotides. Expression and activity of DNMTs is generally restricted to dividing cells and is very high during early development (Szyf et al., 1985, 1991; Monk et al., 1987; Singer-Sam et al., 1990; Goto et al., 1994). DNA methylation can induce long-term transcriptional silencing through direct interference with transcription factor binding. In addition, methylated DNA can counter the transcriptional effects of histone acetylation by recruiting chromatin remodeling enzymes, including histone deacetylases (HDACs), via the action of methyl-CpG binding domain proteins (MBDs) like MeCP2 (Becker et al., 1987; Nan et al., 1997, 1998; Jones et al., 1998; Cross et al., 1997).

DNA methylation has been studied extensively in development and has long been considered a static process following cell differentiation, because typically DNMT expression greatly diminishes once terminal differentiation has occurred (Bestor et al., 1988; Szyf et al., 1985, 1991; Monk et al., 1987; Singer-Sam et al., 1990; Goto et al., 1994; Deng and Szyf, 1999). Because the mammalian brain primarily consists of postmitotic neurons and glial cells that possess relatively low proliferative potential, reports that the adult mammalian CNS possesses high levels of DNMT mRNA and enzymatic activity were unexpected (Monk et al., 1987; Goto et al., 1994; Brooks et al., 1996). Early studies into the function of DNMT in the brain suggested that this enzyme might be involved in DNA repair and neurodegeneration (Brooks et al., 1996; Endres et al., 2000; Fan et al., 2001; Endres et al., 2001). However, recent studies have also implicated misregulation of DNA methylation and DNMTs in such cognitive disorders as schizophrenia, Rett syndrome, and Fragile X mental retardation (Veldic et al., 2004; Amir et al., 1999; Sutcliffe et al., 1992).

To begin investigating a potential role for DNA methylation in the adult CNS, we examined a provocative possibility contrary to the prevailing model of an exclusive role for DNA methylation in development. Thus, we investigated whether DNA methylation regulates memory consolidation in the *adult* CNS via gene-specific control of transcription within the hippocampus.

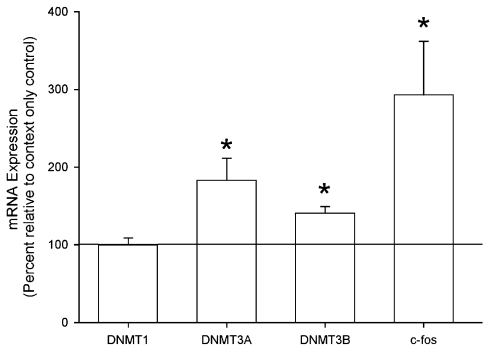

Figure 1. Fear Conditioning Is Associated with an Upregulation of DNMT mRNA

DNMT3A, DNMT3B, and c-*fos* mRNA in area CA1 are upregulated within 30 min of fear conditioning in context-plus-shock animals, relative to context-only controls. *$p < 0.05$. Error bars represent SEM.

RESULTS

DNA Methyltransferase Activity Is Necessary for Memory Formation

We recently characterized the effects of DNMT inhibition on hippocampal synaptic plasticity (Levenson et al., 2006). We found that DNA of the gene *reelin*, which is involved in the induction of synaptic plasticity, exhibits rapid decreases in cytosine methylation when DNMT activity is blocked in acute hippocampal slices. We also found that DNMT inhibition prevents the induction of LTP. These findings suggested that DNA methylation might be dynamically regulated in the adult nervous system and serve as an additional epigenetic mechanism governing memory formation. To pursue this idea, we first investigated whether or not DNMT mRNA levels in the hippocampus are altered by contextual fear conditioning, a hippocampus-dependent associative memory paradigm. Using real-time quantitative PCR, we examined DNMT mRNA levels in the adult rat hippocampus 30 min after training for contextual fear conditioning. We assayed levels of three DNMT subtypes, DNMT 1, 3A, and 3B, as well as the immediate-early gene c-*fos*, which is rapidly induced in the hippocampus by fear conditioning (Melia et al., 1996; Maciejak et al., 2003; Huff et al., 2006). Though there is some overlap in function, DNMT1 has preferential activity for hemimethylated DNA and is traditionally considered a maintenance methyltransferase in DNA replication, while 3A and 3B are responsible for de novo methylation (Siedlecki and Zielenkiewicz, 2006). Animals exposed to the associative context-plus-shock training displayed an increase in DNMT3A and 3B mRNA in area CA1 relative to animals exposed only to the novel context of the fear conditioning chamber (DNMT3A: $t_7 = 2.88$; DNMT3B: $t_7 = 4.56$; c-*fos*: $t_7 = 2.81$; p values < 0.05; Figure 1). These initial findings suggested the intriguing possibility that DNMT activity might be dynamically regulated in the adult CNS in vivo in response to environmental sensory stimulation.

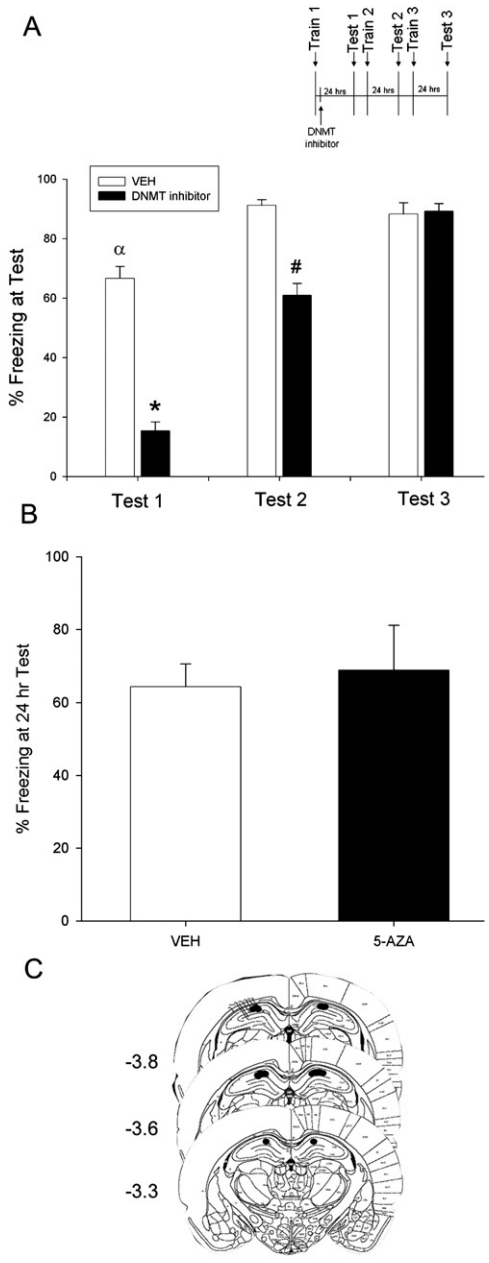

Figure 2. DNMT Inhibition Blocks Memory Consolidation in a Plastic Manner

(A) Intra-CA1 infusion of DNMT inhibitor immediately after contextual fear conditioning training blocked consolidation, as demonstrated by an absence of freezing behavior at the 24 hr test (test day 1). However, memory is formed normally (test day 2) if these same animals are retrained immediately after test 1 and allowed to consolidate the memory in the absence of drug. A third round of training establishes that DNMT inhibitor animals are capable of forming memories equal in strength to vehicle-treated animals (test day 3) ($F_{(5,54)} = 73.08$). * Denotes significantly greater than test day 1 DNMT inhibitor, $p < 0.005$. # Denotes significantly different from all others, $p < 0.005$.

(B) DNMT inhibitor infusions fail to block memory formation if administered 6 hr after training.

(C) Location of needle tips for all intra-CA1 infusions. Diagram represents histology from animals whose behavioral data are depicted in (A) and (B) and Figure 5. Because of the extensive overlap between the infusion needle tips of these animals, not all tip locations are resolvable on this diagram.

Error bars represent SEM.

Because fear conditioning led to an upregulation of hippocampal DNMT mRNA, we next tested the necessity of DNMT activity for memory formation. For this experiment, we infused one of two distinct DNMT inhibitors, 5-aza-deoxycytidine (5-AZA) or zebularine (zeb), directly into area CA1 of the hippocampus immediately after contextual fear conditioning. Infusions were administered *after* training for this hippocampus-dependent task to avoid state-dependent effects of the drug. There were no differences in time spent freezing between the animals infused with 5-AZA and those infused with zeb during the retention tests ($p > 0.05$), nor were there any differences between their respective vehicle groups (0.8% acetate and 10% DMSO; $p > 0.05$). Therefore, the 5-AZA and zeb data were collapsed into one DNMT inhibitor group, as were the two vehicle groups. When memory was assessed 24 hr later (test day 1), animals infused with a DNMT inhibitor (5-AZA or zeb) displayed significantly less freezing than their vehicle-treated (VEH) counterparts ($F_{(1,22)} = 103.9$; $p < 0.001$; Figure 2A), indicating that hippocampal DNMT activity is necessary for memory consolidation.

DNA methylation is not generally considered to be a plastic process; in development, alterations in DNA methylation are essentially permanent. This consideration raised the question of whether or not the effects of DNMT inhibition on the capacity for memory formation are permanent. To address this, we assessed the ability of these same DNMT inhibitor-treated animals to form the fear memory later on, in the absence of the drug. For this experiment, animals treated with DNMT inhibitor or vehicle 24 hr earlier were retrained for contextual fear conditioning immediately after testing on test day 1. Twenty-four hours later, fear memory was again assessed (test day 2). The freezing behavior in vehicle-treated animals on test day 2 was significantly greater than their freezing during test 1 ($p < 0.005$) and lasted for nearly the entire test period (Figure 2A). This result was expected, as this test followed a second training trial for a task in which a single trial is sufficient to form a strong, long-lasting memory. Animals treated with DNMT inhibitor after the first training trial and subsequently retrained on test day 1 showed significantly greater freezing on test day 2 as compared to their performance on test day 1 ($p < 0.005$). This result establishes that the drug infusion did not damage the hippocampus. More importantly, it demonstrates that the effects of DNMT inhibition are not immuteable. Rather, the changes are plastic, allowing the DNA methylation states necessary for memory consolidation to be re-established after transient DNMT inhibition and DNA demethylation.

Interestingly, test day 2 freezing in previously DNMT inhibitor-treated animals was equivalent to the freezing displayed by vehicle-treated animals on test day 1 and

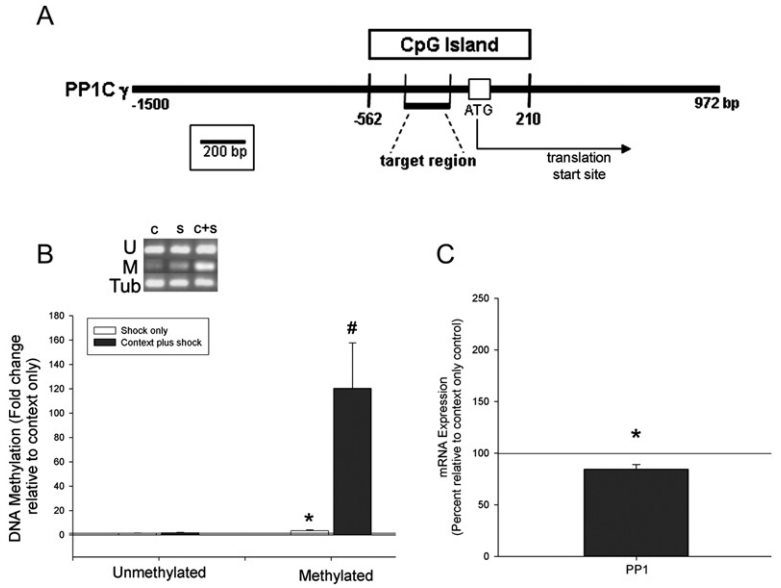

Figure 3. DNA Methylation of a Memory Suppressor Gene Increases with Contextual Fear Conditioning Training

(A) Schematic representation of the location of the methylation changes. Primer sets, as described in Experimental Procedures, were designed to amplify the "target region" within a CpG island located in the *PP1* gene. All base-pair (bp) annotations are relative to the location of the ATG start codon, which represents the translation initiation site. The scale bar represents 200 bp.

(B) Levels of methylated *PP1* have increased, and levels of unmethylated *PP1* have decreased relative to context and shock-only controls in area CA1. *$p < 0.005$ for methylated shock-only relative to context-only. #$p < 0.01$ for methylated context-plus-shock relative to context-only.

(C) Levels of PP1 mRNA in area CA1 are decreased an hour after fear conditioning relative to context-only controls. *$p < 0.05$.

Error bars represent SEM.

slightly, but significantly, less than freezing displayed by vehicle-treated animals on test day 2 (Figure 2). Therefore, the test day 2 freezing behavior of previously DNMT inhibitor-treated animals was equivalent to freezing observed in animals that received one training trial (VEH test day 1), not two (VEH test day 2). This also is consistent with the idea that DNMT inhibition following training prevented consolidation of the fear memory for that trial; that is, there does not appear to be a residual hidden or latent memory for the first training trial in DNMT inhibitor-treated animals.

Finally, we trained all animals a third time, immediately after test 2, to ensure that animals previously treated with a DNMT inhibitor are capable of forming a memory equal in strength to control animals. As depicted in Figure 2A, both groups showed equivalently high levels of freezing on test day 3 ($p > 0.005$).

We next performed a control experiment to ensure that the memory deficit observed at the 24 hr test was truly due to an effect of the drug on memory consolidation and not retrieval. An alternative explanation for the lack of freezing during the test at 24 hr is that the drug had not cleared from the hippocampus between the time of infusion and testing and produced nonspecific effects on retrieval or performance. To test this, we trained animals for fear conditioning and returned them to their home cages. We allowed 6 hr for the memory to consolidate before giving animals intra-CA1 infusions of 5-AZA. The following day (24 hr after training and 18 hr after infusion), we tested the animals for their fear memory. Both vehicle and DNMT inhibitor animals displayed normal fear memory ($F_{(1,11)} = 0.11$, $p > 0.05$; Figure 2B), indicating that the lack of freezing on test day 1 in the previous experiment was due to the effect of DNMT inhibition on consolidation and not retrieval or performance.

Following these experiments, animals were euthanized and histology was performed to confirm the location of the infusion needle tips. The infusion needles effectively targeted area CA1 in all animals (Figure 2C).

Rapid Increases in Methylation Control the Transcription of a Memory Suppressor Gene

To shed further light on the role of DNMTs in memory consolidation, we next looked for direct evidence of altered DNA methylation during memory consolidation. To this end, we employed methylation-specific quantitative real-time PCR to examine methylation changes of a specific target gene known to suppress learning and memory. Inhibition of this memory suppressor gene, protein phosphatase 1 (*PP1*), enhances LTP, the efficacy of associative training, and the maintenance of memory (Blitzer et al., 1998; Jouvenceau et al., 2006; Genoux et al., 2002). For this experiment, we trained a group of animals for fear conditioning, along with context-only and shock-only control groups, and examined the effects of fear conditioning on *PP1* methylation levels (Figure 3A). One hour after contextual fear conditioning training, we observed a dramatic increase in *PP1* gene methylation in the context-plus-shock animals relative to context-only controls ($t_{17} = 3.21$, $p < 0.01$, Figure 3B). Shock-only animals showed a modest increase in *PP1* methylation levels as well ($t_{17} = 3.35$, $p < 0.005$, Figure 3B). The increase in *PP1* methylation produced by fear conditioning directly demonstrates plasticity of DNA methylation in the adult CNS in response to behavioral training. Moreover, this dramatic increase is triggered specifically when the animal forms an association between a novel context and a footshock. In addition, this increase in methylation fits with current models of memory formation, wherein certain memory-suppressing genes must be transcriptionally silenced for

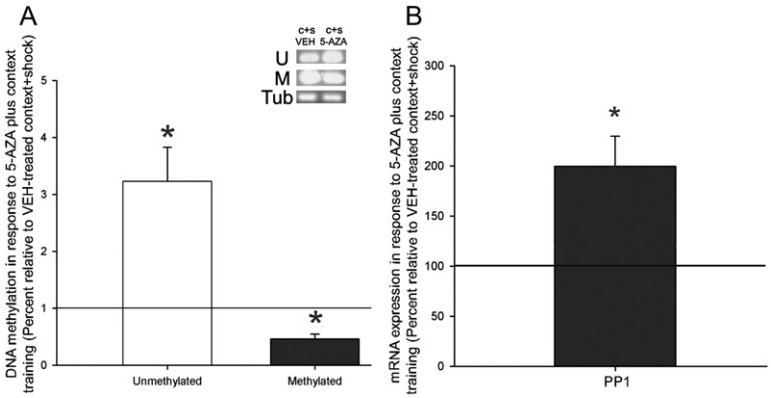

Figure 4. DNMT Inhibition Prevents Suppression of a Memory Suppressor Gene

(A) The increase in *PP1* methylation produced by fear conditioning is blocked by DNMT inhibition. *$p < 0.005$.

(B) The blockade of PP1 methylation by DNMT inhibition results in enhanced PP1 mRNA. *$p < 0.05$.

It is worth noting that the DNMT infusions did not completely block the *PP1* gene methylation induced by conditioning. The most likely reason for this is a technical one. In order to avoid overflow of the DNMT inhibitor into brain regions that neighbor the hippocampus, the infusion volume used most likely did not reach the most rostral portion of area CA1, which was included in the methylation assay.

Error bars represent SEM.

normal memory consolidation to take place (Abel and Kandel, 1998).

We next examined whether or not the increased *PP1* methylation actually resulted in altered transcription of the gene. Using quantitative real-time PCR, we found that context-plus-shock animals had significantly lower levels of PP1 mRNA in area CA1 1 hr after training, as compared to context-only controls ($t_7 = -3.47$, $p < 0.05$, Figure 3C). This finding is consistent with an earlier report of decreased PP1 mRNA in area CA1 of animals that underwent fear conditioning as compared to handled controls (Levenson et al., 2004b).

DNMT Inhibition Prevents the Silencing of a Memory Suppressor Gene

We next looked for confirmatory molecular evidence that intra-CA1 infusions of a DNMT inhibitor following fear conditioning have the intended biochemical effect; that is, decreased levels of DNA methylation at the *PP1* locus. For this experiment, all animals received context-plus-shock pairings. Immediately after training, half of the animals were given infusions of 5-AZA while the other half received vehicle infusions. We then examined *PP1* gene methylation state and observed that 5-AZA attenuated *PP1* methylation within an hour of training and infusion ($t_{11} = -5.72$, $p < 0.001$; Figure 4A) and produced a concomitant increase in *un*methylated *PP1* ($t_{11} = 3.71$, $p < 0.01$; Figure 4A). These findings directly confirm that DNMT inhibitors are effective at blocking fear conditioning-associated increases in gene methylation.

Interestingly, these changes in DNA methylation were accompanied by a 2-fold *increase* in PP1 mRNA ($t_7 = 3.27$, $p < 0.05$; Figure 4B). This finding provides a parsimonious explanation for the mechanism by which DNMT inhibitors block memory formation: they unmask a latent mechanism for memory suppression. Thus, because of PP1's role as a phosphatase and memory suppressor, the increase in PP1 mRNA may, at least in part, explain the lack of memory formation following DNMT infusion.

Rapid Demethylation Controls the Transcription of a Memory Promoting Gene

After observing such dynamic increases in DNA methylation with fear conditioning, we next examined the possibility of the converse reaction occurring, that is, DNA *de*methylation. For this experiment, we investigated methylation of a gene that promotes synaptic plasticity and memory, *reelin*. Specifically, *reelin* enhances LTP induction, and a loss of its function results in a deficit in memory formation (Weeber et al., 2002a; Beffert et al., 2005). We reasoned that if increased methylation occurs at a memory suppressor such as *PP1*, we might observe decreased methylation at a memory promoter, such as *reelin*.

Thus, we assessed the levels of *reelin* gene methylation (Figure 5A) in area CA1 1 hr after training in context-plus-shock trained animals, as well as context- and shock-only control groups. One hour following training, context-plus-shock animals showed a significant reduction in *reelin* gene methylation relative to context-only controls ($t_{15} = -4.94$, $p < 0.001$) and a concomitant increase in *un*methylated *reelin* DNA ($t_{15} = 2.46$, $p < 0.05$). Shock-only animals showed no change in methylation relative to controls (Figure 5B). As with the increase in *PP1* methylation following fear conditioning, this finding is consistent with the accepted function of DNA methylation as a transcriptional silencer. Decreased methylation of the *reelin* gene should result in enhanced transcription of reelin, a gene product that promotes long-term synaptic plasticity and memory formation. We address this more directly in experiments described in the next paragraph. Importantly, the decrease in *reelin* methylation also indicates the presence and activity of not only DNMTs during memory consolidation, but also an as yet unidentified DNA demethylase that demethylates DNA in an activity-dependent fashion.

We next examined whether or not the decreased *reelin* methylation actually resulted in enhanced transcription of the gene. Using quantitative real-time PCR, we found that context-plus-shock animals had significantly greater levels of reelin mRNA in Area CA1 one hour after training,

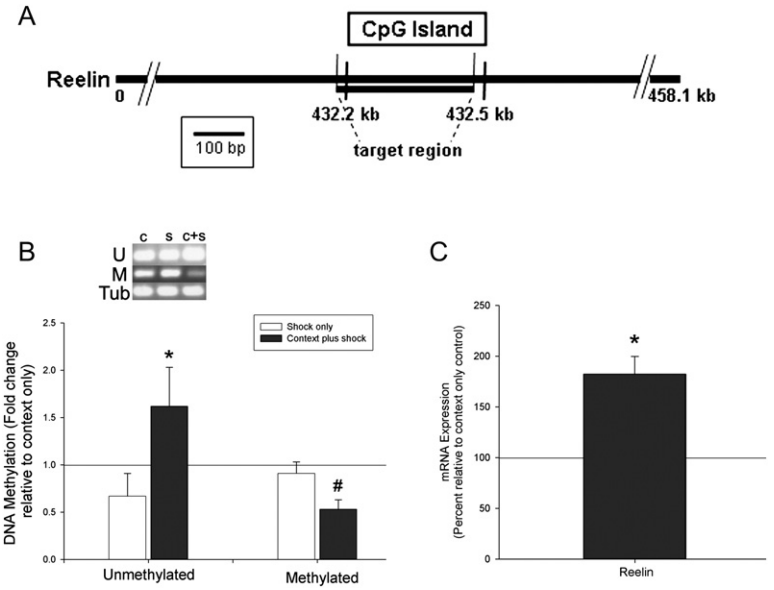

Figure 5. Contextual Fear Conditioning Training Leads to the Demethylation of *reelin* DNA

(A) Schematic representation of the location of the methylation changes. Primer sets, as described in Experimental Procedures, were designed to amplify the "target region" within a CpG island located in the *reelin* gene. All basepair (bp) annotations are relative to the start of the promoter. The scale bar represents 100 bp.

(B) An hour after fear conditioning, levels of methylated reelin have decreased and levels of unmethylated reelin have increased relative to context and shock-only controls in area CA1. *$p < 0.05$ for unmethylated comparisons. #$p < 0.001$ for methylated comparisons.

(C) Levels of reelin mRNA in Area CA1 are increased an hour after fear conditioning relative to context only controls. *$p < 0.005$.

Error bars represent SEM.

as compared to context-only controls ($t_7 = 4.73$, $p < 0.005$; Figure 5C). This finding is consistent with an earlier report of increased reelin mRNA in area CA1 of animals that underwent fear conditioning as compared to handled controls (Levenson et al., 2004b).

DNMT Inhibition Leads to the Further Demethylation of Reelin

To further confirm the efficacy of the DNMT inhibitor at decreasing methylation levels, we examined the effect of DNMT inhibition in conjunction with fear conditioning on *reelin* methylation. As expected, levels of methylated *reelin* DNA were further decreased by 5-AZA beyond the decrease induced by the fear conditioning training itself (methylated *reelin*: $t_{11} = -5.83$, $p < 0.001$; unmethylated *reelin*: $t_{11} = 3.25$, $p < 0.01$; Figure 6A). Levels of reelin mRNA reflected the alterations in methylation induced by DNMT inhibition: 5-AZA-infused animals showed levels of reelin mRNA above and beyond those induced by fear conditioning alone ($t_7 = 2.59$, $p < 0.05$; Figure 6B). This result demonstrates that environmental training plus DNMT inhibition have additive effects on *reelin* methylation and gene expression, supporting a positive role for *reelin* gene *de*methylation in controlling reelin gene expression.

It is possible that DNMT inhibitors are capable of affecting transcription when the system is at rest and do not require the associative training of fear conditioning in order to trigger altered gene expression. To investigate this, we administered intra-CA1 infusions of 5-AZA or vehicle to animals taken directly from the home cage. Immediately after the infusions, animals were returned to their home cage and were euthanized an hour later. There was no difference in either reelin or PP1 mRNA between the 5-AZA and vehicle-infused groups (Reelin: $t_3 = -0.092$; PP1: $t_3 = 0.127$; p values > 0.05), indicating that application of a DNMT inhibitor is not sufficient to alter transcription in a system "at rest." This leads to the interesting conclusion that inhibition of DNMT activity by itself is not sufficient to regulate *reelin* and *PP1* gene expression, but rather that DNA methylation and demethylation operate in concert with additional mechanisms to regulate transcription.

In addition to *reelin*, we also examined the effect of DNMT inhibition on the expression of DNMT1 and c-*fos* following fear conditioning. Expression of c-*fos*, an immediate-early gene that serves as a reporter of cellular activation, was further augmented by DNMT inhibition (Figure 7) above and beyond the 2-fold increase produced by associative training alone (Figure 1). The effect of 5-AZA plus training is a further 3-fold increase above this level ($t_7 = 3.63$, $p < 0.01$). This result provides a further example, in addition to *reelin*, of the capacity of altered DNA methylation to regulate gene transcription in the adult nervous system. Interestingly, DNMT1, a gene that is not upregulated by fear conditioning, remains unaltered when training is coupled with 5-AZA treatment (Figure 7; $p > 0.05$). These data with c-*fos* and DNMT1 further demonstrate that transcriptional regulation by DNA methylation is gene specific, and the result with c-*fos* provides a second example, in addition to *reelin*, of DNA methylation interacting with associative environmental stimuli in regulating gene transcription in the hippocampus.

DNA Methylation Changes in the Hippocampus Are Highly Dynamic

The ability of animals to learn the fear conditioning task 24 hr after DNMT inhibitor infusions (Figure 2) suggests that changes to DNA methylation in the adult nervous system are not necessarily permanent, but rather can be dynamic and reversible. To more directly investigate this possibility, we examined *reelin* and *PP1* gene methylation levels 24 hr after training. For this experiment, we trained animals for fear conditioning and returned them to their

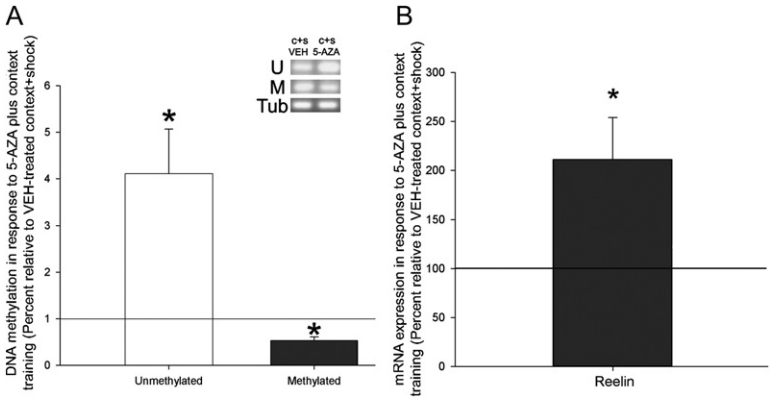

Figure 6. DNMT Inhibition Drives the Further Demethylation of the *reelin* Gene

(A) Intra-CA1 DNMT inhibition decreases methylated *reelin* beyond that produced by fear conditioning. *p < 0.01.

(B) DNMT inhibition results in enhanced reelin mRNA above that produced by fear conditioning. *p < 0.05.

Error bars represent SEM.

home cages. We then euthanized the animals 24 hr later without administering a memory test. Interestingly, the methylation levels of both *reelin* and *PP1* had returned to baseline control levels within a day of training (p values > 0.05, Figure 8). This indicates that not only are the DNA methylation changes following training rapid but they are also surprisingly dynamic. This finding further supports a role for plasticity of DNA methylation as a transcriptional regulator involved in memory formation in the hippocampus. Importantly, these findings contrast with what would be expected based on developmental studies of DNA methylation, wherein changes in gene methylation state are essentially permanent.

DISCUSSION

An enigma facing the learning and memory field relates to the ability of memories to remain stable in the face of constant molecular turnover. In 1984, Francis Crick postulated that the required stability might be based on the self-perpetuating modification of specific proteins and

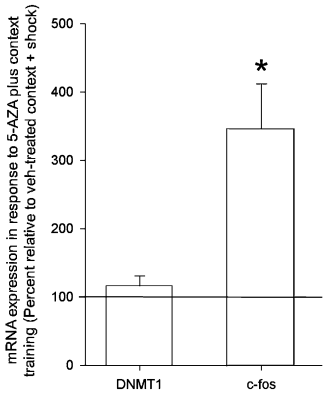

Figure 7. Genes Must Be Activated by Fear Conditioning for DNMT Inhibition to Alter Their Regulation

In area CA1, levels of c-*fos* but not DNMT1 mRNA are increased 1 hr after fear conditioning, relative to context-only controls. *p < 0.01. Error bars represent SEM.

modeled his hypothesis on the known mechanisms for perpetuation of DNA methylation (Crick, 1984). Fifteen years later, Robin Holliday expanded on this theory, proposing that specific sites in the DNA of neurons involved in memory might exist in alternative methylated or nonmethylated states (Holliday, 1999). This was based on findings from studies in the developmental field on gene imprinting and cell differentiation. These developmental studies indicated that methylation states are capable of providing the needed complexity to control overall patterns of gene transcription and are long-lasting, allowing them to provide the memory necessary to maintain a cellular phenotype after differentiation. This led Holliday to predict that perhaps DNA methylation could provide the same mechanisms for memory storage.

Now, over 20 years since Crick's initial postulation, we have data to support the idea that DNA methylation does in fact play an important role in learning and memory. To our knowledge, this study is the first to present evidence that DNA methylation, once thought to be a static process after cellular differentiation, is not only dynamically regulated in the adult nervous system but also plays an integral role in memory formation. Our results indicate that DNA methylation, like modification of chromatin, is an epigenetic mechanism that has been co-opted by the adult CNS to serve as a crucial step in the transcriptional regulation underlying memory consolidation. However, in contrast to the Crick/Holliday conjecture, DNA methylation in the adult hippocampus does not appear to play a role in long-term memory storage, as the changes reverse within 24 hr. This attribute is, however, consistent with the role of the hippocampus as a structure contributing to memory consolidation but not memory storage. It will be interesting to determine if the Crick/Holliday mechanism plays a role in perpetuating long-term changes in adult neurons in the cortex, at known sites of long-term memory storage.

We have shown using direct molecular methods that DNA methylation levels are rapidly and dynamically regulated in the hippocampus following the associative training paradigm of contextual fear conditioning. We also found that mRNAs for the de novo methyltransferases, DNMT3A and 3B, are upregulated in area CA1 of the

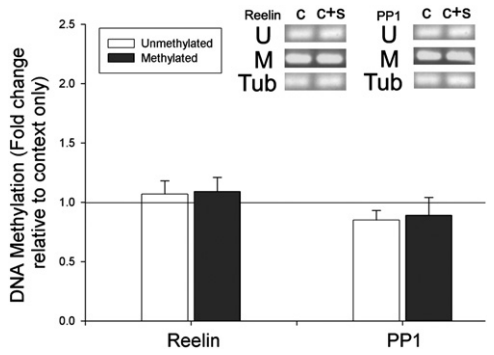

Figure 8. Methylation Levels Are Dynamically Regulated in the Hippocampus

Levels of reelin and PP1 methylation in context-plus-shock animals return to baseline within 24 hr of training. Error bars represent SEM.

hippocampus following fear conditioning and that pharmacologically inhibiting DNMT activity blocks normal memory consolidation. Importantly, animals are able to form association memories normally once the DNMT inhibitor has cleared from the hippocampus, suggesting that the alterations in DNA methylation produced by the inhibitors are not permanent, but rather are subject to reversal by ongoing cellular processes. Overall, these findings indicate an unanticipated level of plasticity of DNA methylation in the adult CNS. They are also consistent with a model wherein covalent modification of DNA is a dynamic process in the adult CNS subject to regulation by an ongoing interaction between environmental signals and maintenance processes resident in the cell.

In addition, we show that through bidirectional regulation of methylation levels, the hippocampus has adopted a method for the specific regulation of genes. Intuitively, it seems that some genes must be activated and others silenced in order for normal memory formation to occur. In line with this thinking, we have observed that increased methylation of *PP1* following fear conditioning acutely silenced the gene. Simultaneously, decreased methylation of *reelin* releases the gene from transcriptional repression, resulting in increased production of the gene's mRNA. Inhibition of PP1 has been shown to not only enhance learning but also to increase phosphorylation of the AMPA receptor subunit GluR1, Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), and cAMP response element binding protein (CREB) (Genoux et al., 2002). Therefore, silencing the *PP1* gene via methylation should allow phosphorylation of crucial receptors, kinases, and transcription factors during the memory-consolidation period. The *reelin* gene product promotes synaptic plasticity and long-term memory formation (Weeber et al., 2002a) and presumably promotes memory consolidation in concert with suppression of *PP1*. Importantly, the methylation levels of both *PP1* and *reelin* return to baseline within 24 hr of training, indicating just how dynamic the changes are.

We have also demonstrated that the DNMT inhibitor 5-AZA is an effective demethylating agent in the adult CNS, as it leads to the *further* demethylation of *reelin* beyond what is induced by fear conditioning alone. Importantly, in terms of the DNMT inhibitors' effects on memory, the loss of *PP1* methylation produced by DNMT inhibition is accompanied by an *increase* in PP1 transcription. This likely accounts, at least in part, for the lack of memory consolidation in DNMT-inhibited animals. Interestingly, silencing *PP1* may have important effects during normal memory consolidation for transcriptional regulation by CREB, through PP1's ability to complex with HDAC1. Using HEK293 cells, Canettieri and colleagues have demonstrated that an HDAC1-PP1 complex represses CREB activation under basal conditions and dephosphorylates CREB to return the system to baseline after a stimulus (Canettieri et al., 2003). This suggests that repression of PP1 during the consolidation period is crucial in order to provide phosphorylated CREB with the capacity to recruit CBP to the promoter, at which time histones become acetylated and help to drive the transcription of particular genes. By inhibiting the methylation of *PP1* with a DNMT inhibitor following fear conditioning, we increased levels of PP1 mRNA present in the hippocampus. It is possible that the resulting increase in PP1 cut short the activation of CREB, interfering with the transcriptional regulation of CREB-regulated genes necessary for memory formation.

As we mentioned earlier, a number of intracellular events must occur in the hippocampus in order for memories to consolidate. This begins with NMDA receptor activation and the eventual translocation of ERK to the nucleus where it has a variety of effects, including activation of the transcription factors CREB and ets-like gene-1 (Elk-1) (Impey et al., 1998; Roberson et al., 1999). This leads to alterations in gene transcription that are critical for long-term memory formation. The current findings may help to fill in the gap between transcription factor activation and gene transcription. Figure 9 depicts a model of how alterations in DNA methylation may be driving long-term memory formation. An as yet unknown signaling pathway targets the nucleus and activates demethylases and DNMTs. This results in the demethylation of positive regulators of memory, such as *reelin*. HATs are then free to acetylate demethylated genes, releasing them from the transcriptional silencing induced by methylation. This leads to transcriptional activation of *reelin* and, likely, other memory-enhancing genes. Simultaneously, DNMTs target negative regulators of memory, such as *PP1*, for transcriptional silencing. DNMT activation results in increased methylation of the *PP1* gene, which can lead to the recruitment of MBDs to this locus. MBDs can recruit HDACs, leading to deacetylation and transcriptional silencing of *PP1* and potentially other memory-suppressing genes. The two arms of this model depict opposing epigenetic actions, which nevertheless work in concert to achieve the same end—long-term memory formation.

The fact that DNA methylation is important for memory consolidation fits with the emerging understanding that the nervous system has co-opted epigenetic mechanisms for the formation and storage of memories in the adult. To

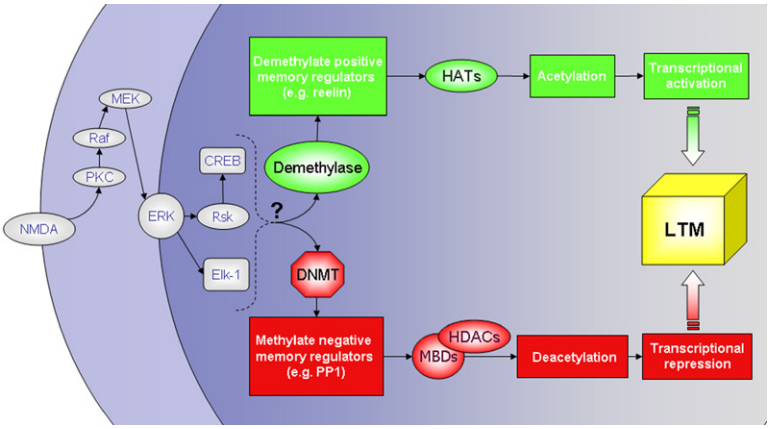

Figure 9. Schematic Representation of the Role DNA Methylation May Be Playing in the Transcriptional Regulation of Memory Formation in the Hippocampus

Note: The receptors, kinases, and transcription factors depicted in gray play established roles in hippocampal memory consolidation. However, the present study does not address the potential link between these proteins and the DNA methylation we report here to be important for memory formation.

date, there is strong evidence supporting a role for chromatin modifications in memory consolidation (Swank and Sweatt, 2001; Guan et al., 2002; Huang et al., 2002; Levenson et al., 2004a, 2006; Korzus et al., 2004; Alarcon et al., 2004; Wood et al., 2005; Kumar et al., 2005; Chwang et al., 2006). The findings presented here, however, are particularly significant because they are in such stark contrast to the current understanding of the role of cytosine methylation in development. It is thought that DNA methylation is crucial for normal development and that embryonic methylation patterns are maintained in perpetuity postnatally—only to be perturbed in cases like cancer in which abberant hypomethylation occurs and a subsequent loss of transcriptional regulation of these genes (Santos et al., 2005). The results of our study, however, indicate that, at least within the hippocampus, DNA methylation levels can be rapidly and dynamically altered by environmental stimuli that induce associative learning. This finding necessitates a shift in the way we think about cellular roles for DNA methylation.

Our findings also complement recent discoveries concerning epigenetic aberrations observed in cancer and cognitive disorders. Just as we observed during memory consolidation, cancer research has revealed bidirectional DNA methylation-dependent regulation of genes. For example, tumorogenisis appears to be driven by global hypomethylation working in concert with hypermethylation of a specific subset of genes (Luczak and Jagodzinski, 2006). In addition, recent studies have implicated misregulation of DNA methylation in a number of cognitive disorders, including several autism spectrum disorders and schizophrenia (reviewed in Weeber et al., 2002b; Grayson et al., 2006). Fragile X mental retardation results from abnormal trinucleotide expansion, which leads to decreased gene expression through aberrant DNA methylation and restrictive chromatin structure (reviewed in Weeber et al., 2002b). Rett syndrome is associated with mutations in MeCP2, one of the MBDs recruited by methylated DNA that contributes to gene silencing (Amir et al., 1999; Collins et al., 2004). And a recent study identified an overlapping pathway of gene dysregulation within 15q11-13 in Rett, Angelman syndrome, and autism and implicated MeCP2 function in all three through studies of *MeCP2*-deficient mice and human Rett, Angelman syndrome, and autism brains (Samaco et al., 2005).

In addition, hypermethylation of the reelin gene is a rapidly emerging hypothesis as a potential basis for schizophrenia, a disorder marked by a variety of cognitive deficits. In the cortex of schizophrenic patients, there is typically a 50% reduction in reelin mRNA, an effect associated with aberrant methylation of the gene (Chen et al., 2002). The findings presented here may provide an important and relevant piece of data to the schizophrenia field, as they provide evidence that *reelin* methylation is subject to modulation in response to experience and environmental stimuli. In addition, the current findings indicate that not all alterations in DNA methylation are aberrant; rather, some changes naturally occur during normal memory formation.

In this study we report that DNMT inhibition prevents memory consolidation. However, because both 5-AZA and zebularine are potent DNMT inhibitors, affecting all three subtypes (DNMT1, 3A, and 3B; Weisenberger et al., 2004; Marquez et al., 2005), we are unable to determine which of the subtypes is important for hippocampal memory formation. We also report an increase in the mRNA of the two de novo methyltransferases (3A and 3B) within a half hour of fear conditioning training. Perhaps both DNMT3A and 3B work in concert to bring about de novo methylation of the necessary genes, such as *PP1*, and transcriptionally silence these genes, aiding in memory formation.

An additional implication of the current findings is that a DNA demethylase must exist and be regulated in the adult CNS. Identification of a demethylating enzyme is one of the more intriguing and controversial aspects of the current DNA methylation literature. At present there is no clearly identified DNA demethylase; however, at least two candidates are identifiable based on current literature. Detich and colleagues have reported demethylase activity of an MBD in vitro (Detich et al., 2002)—overexpression of MBD2 in cell culture induced demethylation at a number of

sites within the promoter region of a reporter gene. In addition, Agius and colleagues have proposed ROS1 as an active DNA demethylating agent in plants (Agius et al., 2006). However, the mammalian DNA demethylase is completely mysterious at present. In fact, to our knowledge, our finding of reduced *reelin* methylation following fear conditioning is the first demonstration of nonpathological DNA demethylation occurring in vivo. Our data, therefore, imply the existence of a signaling cascade in the adult CNS controlling the demethylation of DNA in a dynamic fashion and suggest that perhaps the demethylase may be identifiable in CNS-specific tissue.

Future studies will investigate upstream molecules that may regulate DNMT and DNA demethylase activity. One upstream candidate regulator is ERK, which plays an integral role in hippocampal memory formation (Sweatt, 2004). In addition, our lab has recently demonstrated that ERK is upstream of two chromatin modifications, histone acetylation and phosphorylation (Levenson et al., 2004a; Chwang et al., 2006). Thus, ERK may target DNMTs and/or demethylases as a means of translating a general signal received at the postsynaptic membrane during associative learning to specific genes within the nucleus.

We are also interested in the ways in which DNA methylation interacts with chromatin modifications. We have reported preliminary evidence that in acute hippocampal slices, DNMT blockade prevents protein kinase C (PKC)-induced *increases* in histone H3 acetylation (Levenson et al., 2006). This is not the result one would predict if DNA methylation were operating only to recruit HDACs and thus silence genes by that mechanism. Therefore, although our model in Figure 9 depicts a unidirectional regulation of histone acetylation by DNA methylation, the interactions are likely far more complicated. In particular, it will be of interest to see if DNMT blockade similarly interferes with the histone H3 acetylation associated with fear conditioning and if altering acetylation levels has any effect on DNA methylation levels.

The specific roles of covalent chemical modification of DNA in memory processes in the adult nervous system are sure to be complex, likely warranting decades of future research. Nevertheless, the findings presented here indicate the importance of dynamic regulation of DNA methylation in behavioral changes brought about by the perception of environmental stimuli.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley rats weighing 250–300 g were used for all experiments. Rats were housed under 12:12 light/dark cycles, with food and water available ad libidum. All procedures were performed in accordance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee and with national regulations and policies. All animals used for behavioral experiments were handled for 3–5 days prior to the start of behavioral conditioning.

Behavioral Procedures

For contextual fear conditioning, animals were placed into the training chamber and allowed to explore for 2 min, after which they received an electric shock (1 s, 0.5 mA). The 2 min/1 s shock paradigm was repeated for a total of three shocks. After the final shock, animals remained in the training chamber for an additional 1 min. Context-only control animals were exposed to the fear conditioning context during the training period, but received no shock. Shock only animals received three consecutive 1 s, 0.5 mA foot shocks while being held by the experimenter. The control shocks occur quickly enough that the animals do not form an association with the context, which we verified by testing a subset of these shock-only animals 24 hr later for their freezing response. When appropriate, intra-CA1 infusions of the DNMT inhibitors, ZEB or 5-AZA, were performed immediately posttraining. For experiments in which CA1 tissue was to be used for biochemistry or quantitative real-time PCR, a subgroup of animals was allowed to survive and was tested for retention of the fear memory 24 hr later to confirm the memory effects.

Cannula Implantation

For stereotaxic surgery, rats were anesthetized with ketamine and xylazine and secured in a Kopf stereotaxic apparatus. Bilateral stainless-steel guide cannulae (26G; Plastics One, Roankoke, VA) were aimed at area CA1 of the hippocampus (AP: −3.6 mm relative to bregma; ML: ± 1.7 mm; DV: −2.6 mm from skull; Paxinos and Watson, 1998). Clearance through the guide cannulae was maintained with 33G obdurators (Plastics One) cut to project 1 mm beyond the tip of the guide. Animals were habituated to dummy cannula removal and given 5 days of recovery and handling before the start of behavioral conditioning. To ensure accurate cannula placement, brains were collected from those animals given both fear conditioning training and a retention test. 40 μm sections were collected through area CA1 and stained with cresyl violet to verify the location of the infusion needle tips. Infusion needle tips were found to be located well within area CA1 in all cannulated animals (Figure 2C).

Drugs

Zebularine (Calbiochem) was dissolved in 10% DMSO and diluted to a concentration of 600 ng/μl in sterile saline. 5-aza-deoxycytidine (VWR) was dissolved in 0.8% acetate and diluted to a concentration of 200 ng/μl in sterile saline.

Intra-CA1 infusion

All drugs were infused at a rate of 0.25 μl/min for 2 min. Infusion needles were left in place for 1 min after the infusion to allow for diffusion of the drug.

Isolation of Area CA1

For isolation of area CA1 from whole brain, brains were immersed in oxygenated (95%/5% O_2/CO_2) ice-cold cutting saline (CS; in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 7 MgCl2, 5 glucose, 0.6 ascorbate) immediately after rapid decapitation and removal of the brain. Area CA1 was dissected away from other hippocampal subfields under a dissecting scope and immediately frozen on dry ice and stored at −80°C overnight.

Real-Time Reverse Transcription Quantitative PCR

RNA extractions were performed on area CA1 tissue from animals that were euthanized 30 min (for DNMTs and c-*fos*) or 1 hr (reelin and PP1Cγ) after fear conditioning training. RNA was isolated from area CA1 tissue using Trizol (Invitrogen). RNA samples were further purified with $CHCl_3$ and concentrations were determined spectrophotometrically. Real-time reverse transcription quantitative PCR was performed in one step using commercially available reagents (iScript one-step supermix, Bio-Rad) and Taqman probes for β-tubulin 2B, DNMT1, DNMT3A, DNMT3B, c-*fos*, reelin, and PP1C γ (Applied Biosystems, Foster City, CA). All probes were designed to span exon boundaries, ensuring amplification of only mRNA. Equal amounts of RNA were analyzed in triplicate for each probe used; equal loading was confirmed by amplification of β-tubulin 2B. Ct values were chosen in the linear range of amplification, and the comparative Ct method was used to

calculate differences in gene expression between samples (Livak and Schmittgen, 2001; Pfaffl, 2001).

DNA Methylation Assay

DNA purification (Wizard genomic DNA purification kit; Promega, Madison, WI) was performed on area CA1 tissue from animals that were euthanized 1 hr after fear conditioning training. Purified DNA was then processed for bisulfite modification (CpGenome DNA modification kit; Chemicon). Quantitative real-time PCR was used to determine the DNA methylation status of the *reelin* and *PP1C* γ genes. Methylation-specific PCR primers were designed using Methprimer software (available at www.urogene.org/methprimer/).

Detection of unmethylated reelin DNA was performed using the following primer: forward (5′-TGTTAAATTTTTGTAGTATTGGGG ATGT-3′) and reverse (5′-TCCTTAAAATAATCCAACAACACACC-3′). Detection of methylated reelin DNA was performed using the following primer: forward (5′-GG TGTTAAATTTTTGTAG TATTGGGGAC-3′) and reverse (5′-TCCTTAAAATAAT CCAACAACACGC-3′). Detection of unmethylated PP1 DNA was performed using the following primer: Forward (5′-GAGGAGAGTTTGGTGTTTATAA GATGGT-3′) and reverse (5′-TCC TCCAAAAACTCAACTCAAACAA-3′). Detection of methylated PP1 DNA was performed using the following primer: Forward (5′-GGA GAGTTTGGTGTTTATAAGA TGGC-3′) and reverse (5′-CGAA AACT CGACTCGAA CGA-3′). Samples were normalized to β-tubulin 4 using the following primer: forward (5′GGAGAGTAAT ATGAATGATTTGG TG-3′) and reverse (5′-CATCTCCAACTTTCCCTAACCTACTT AA-3′). PCRs were performed in a total volume of 20 μl, consisting of 2 μl of bisulfite-modified DNA, 10 μl of iQ SYBR Green Supermix (BioRad), 1 μl of primer, and 7 μl of DepC H_2O. Reactions were performed in an iQ5 iCycler real-time PCR system (BioRad). To further verify specificity of the final product, 10 μl of the amplified products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light. Quantitative PCR was run three times with each sample. For every quantitative PCR, samples were assayed in triplicate and the Ct value for each sample was chosen in the linear range. Samples were normalized to β-tubulin 4, and the comparative Ct method was used to calculate differences in gene expression between samples.

Statistical Analysis

One-sample t tests were used to assess DNMT mRNA levels, as well as changes in the methylation state of reelin and PP1. One-way analysis of variance was used to analyze all other data. The Tukey-Kramer post hoc test was used when necessary. Significance was set at $p \leq 0.05$ for all tests.

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