EVIDENCE THAT DNA (CYTOSINE-5) METHYLTRANSFERASE REGULATES SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS

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DNA (cytosine-5) methylation represents one of the most widely used mechanisms of enduring cellular memory. Stable patterns of DNA methylation are established during development, resulting in creation of persisting cellular phenotypes. There is growing evidence that the nervous system has co-opted a number of cellular mechanisms used during development to subserve the formation of long-term memory. In this study, we examined the role DNA (cytosine-5) methyltransferase (DNMT) activity might play in regulating the induction of synaptic plasticity. We found that the DNA within promoters for reelin and brain-derived neurotrophic factor, genes implicated in the induction of synaptic plasticity in the adult hippocampus, exhibited rapid and dramatic changes in cytosine methylation when DNMT activity was inhibited. Moreover, zebularine and 5-aza-2-deoxycytidine, inhibitors of DNMT activity, blocked the induction of long-term potentiation at Schaffer-collateral synapses. Activation of protein kinase C (PKC) in the hippocampus decreased Reelin promoter methylation and increased DNMT3A gene expression. Interestingly, DNMT activity is required for PKC-induced increases in histone H3 acetylation. Considered together, these results suggest that DNMT activity is dynamically regulated in the adult nervous system, and that DNMT may play a role in regulating the induction of synaptic plasticity in the mature CNS.

DNA (cytosine-5) methyltransferases (DNMT) are a family of enzymes that catalyze the methylation of cytosine residues (1-5). Many biological processes including imprinting, differentiation, X-chromosome inactivation and long-term transcriptional regulation involve cytosine methylation, a covalent modification of DNA (6,7). Tissue-specific patterns of DNA-methylation are established early during development as a consequence of cellular differentiation (8,9). Expression and activity of DNMT is generally restricted to dividing cells, and is very high during early development (5,10-13). In most cell types DNMT expression diminishes greatly once terminal differentiation occurs (5,10-14).

The mammalian brain consists primarily of post-mitotic neurons and glial cells that possess relatively low proliferative potential. In addition, there are small populations of stem cells in various regions of the brain that have the potential to develop into new neurons (15). Therefore, reports that the adult central nervous system (CNS) possesses relatively high levels of DNMT mRNA and enzyme activity were surprising (5,13,16). Early studies into the function of DNMT in the brain suggested that this enzyme might be involved in DNA repair and neurodegeneration.
(16-19). Important recent studies also have implicated misregulation of DNMT specifically or DNA methylation in general in such cognitive disorders as Schizophrenia, Rett syndrome and Fragile X mental retardation (20-22).

Epigenetics refers to a set of self-perpetuating post-translational modifications of DNA and nuclear proteins that produce lasting alterations in chromatin structure as a direct consequence, and lasting alterations in patterns of gene expression as an indirect consequence. In a previous study (23), we investigated the hypothesis that neurons have co-opted the epigenetic processes used to form “cellular” memories during development to subserve induction of long-term synaptic plasticity and consolidation of long-term memory (24). We found evidence for regulation of histone acetylation by contextual fear conditioning in the hippocampus, suggesting that at least one form of epigenetic mark is regulated during formation of long-term memory in mammals (23). Other studies have implicated the histone acetyltransferase CREB binding protein (CBP) in formation of hippocampus-dependent long-term memory (25-27). Together these studies suggest that epigenetic modulation of the genome is a necessary component to formation of long-term memory.

Methylation of DNA represents another epigenetic tag. DNA methylation has been shown to induce long-term changes in gene expression through direct interference with transcription factor binding and recruitment of chromatin remodeling enzymes via the action of methyl-CpG binding proteins (28-32). Thus, DNA methylation could have lasting effects on neuronal gene expression and overall functional state. We hypothesize that direct modification of DNA, in the form of DNA (cytosine-5) methylation, is another epigenetic mechanism for long-term information storage in the nervous system. In support of this hypothesis, previous studies have observed decreases in methylation of BDNF3 (rat) and BDNF4 (mouse) promoters in response to depolarization (33,34).

In the studies described below we further tested our hypothesis that epigenetic mechanisms regulate memory formation, by determining the effects of DNA methyltransferase inhibitors on synaptic plasticity. Synaptic plasticity is the leading candidate cellular mechanism for storage of memory in vivo. Long-term potentiation (LTP) of hippocampal Schaffer-collateral synapses has received a great deal of experimental attention, as this form of synaptic plasticity utilizes many of the same mechanisms involved in consolidation of long-term memory (35). Successful formation of LTP requires engagement of NMDA-receptors, activation of the Ras-MEK-ERK signaling cascade, and ultimately post-translational modifications of histones that mediate a transcriptional program resulting in lasting changes in neuronal function (23,25-27,36). Because DNA methylation and histone post-translational modification are intimately associated, we speculated that DNMT activity might regulate synaptic plasticity mechanisms in the CNS. We found that inhibition of DNMT alters DNA methylation within the promoter region of both reelin and BDNF1, indicating that acute regulation of DNA methylation occurs in the adult nervous system. Treatment with phorbol-12,13-diacetate (PDA) induced significant regulation of DNMT3A, suggesting that the PKC signaling pathway regulates the expression of DNMT genes. Moreover, inhibition of DNMT blocked induction of LTP, suggesting that DNMT activity is required for induction of synaptic plasticity. Finally, we provide evidence that PKC-mediated changes in histone H3 acetylation are sensitive to DNMT activity. These findings provide the first evidence for DNA (cytosine-5) methylation in dynamic signaling in the adult nervous system, and suggest that DNMT activity is required for normal hippocampal synaptic plasticity.

MATERIALS AND METHODS

Animals. Young adult (4-8 weeks) male C57BL/6J mice were used for all experiments. Mice were housed under 12:12 Light:Dark cycles, with food and water available ad libitum. All procedures were performed in accordance with the Baylor College of Medicine Institutional Animal Care and Use Committee and with national regulations and policies.

Hippocampus slice preparation. Animals were sacrificed by cervical dislocation, followed by decapitation. The brain was immersed in oxygenated (95%/5% O2/CO2) 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) prior to isolation of the hippocampus. Transverse slices (400 µm) were prepared with a Vibratome (Vibratome 1000, The Vibratome Co., St. Louis, MO). During isolation, hippocampal slices were stored in oxygenated ice-cold CS. After isolation, hippocampal slices were equilibrated in a mixture of 50% CS and 50% artificial cerebrospinal fluid (ACSF [in mM]: 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 25 glucose) at room temperature (RT) for 45 min prior to use.

Pharmacologic stimulation of hippocampal slices. Transverse hippocampal slices, pooled from 6 – 8 animals and randomized, were incubated in oxygenated ACSF (32°C) for 1 h prior to treatment. For DNA methylation assays, slices were treated with either Zebularine (100 µM, 40 min), phorbol-12,13-diacetate (PDA, 3 µM, 20 min), or vehicle (0.5% DMSO). For experiments investigating regulation of DNMT gene expression, slices were treated with either vehicle (0.1% DMSO) or PDA (3 µM) for 45 min. For DNMT inhibition experiments, slices treated with either vehicle (0.5% DMSO) or Zebularine (100 µM), or vehicle (0.001% CH₃COOH) or 5-aza-2-deoxycytidine (30 µM) for 40 min followed by incubation with either vehicle (0.1% DMSO) or PDA (3 µM) for 20 min. Immediately after all treatments, Area CA1 was isolated in ice-cold CS. For DNA methylation assays and experiments measuring DNMT gene expression, tissue was immediately frozen on dry ice. For experiments measuring histone acetylation, tissue was immediately processed for histone extraction.

DNA methylation assay. DNA was isolated from hippocampal tissue, purified (Wizard genomic DNA purification kit, Promega, Madison, WI), and processed for bisulfite modification (CpGenome DNA modification kit, Chemicon, Temecula, CA). Quantitative real-time PCR was used to determine the DNA methylation status of the Reelin and BDNF1 promoter. Methylation-specific PCR primers were designed using Methprimer software (http://www.urogene.org/methprimer/). One set of primers targeted a large CpG island present in the reelin promoter (see also ref 37), and another set of primers targeted two CpG islands detected in silico in the promoter upstream of exon I in the mouse BDNF gene. Detection of unmethylated DNA in the reelin promoter was performed using the following primers: Region 1: forward, 5'-GGTTGTTAGTGGTGTTAGATAAAAGAATAGT-3' and reverse, 5'-CCCCAAAAACAAAAACTACTCAAC-3'; Region 2: forward, 5'-TTTTGTTGAGGAATTTTTAGTAATG-3' and reverse, 5'-AAAAACCATCATAAACTTCTCAAC-3'. Detection of unmethylated DNA in the BDNF promoter was performed using the following primers: CpG island 1: forward, 5'-GGGTAGTGATTTTGGGGAGGAAGTAT-3' and reverse, 5'-CAACCTCTATACACCAACTAAATCCACC-3'; CpG island 2: Forward 5'-TGGGGAAATTTGAAAAGTAGTGT-3' and reverse, 5'-CACACAAAAACAAAAACAAAAAACC-3'. PCR reactions were performed in a total volume of 20 µl, consisting of [µL]: 2 bisulfite modified DNA, 10 iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 1 primer (18 µM), 7 DEPC-treated ddH₂O. Reactions were performed in a Chromo4 real-time PCR system (Bio-Rad, Hercules, CA) using the following cycling conditions: 95 °C for 3 min, 40 cycles of 95 °C for 15 sec, 60 °C for 1 min, and either 77.5 °C (reelin, Region 1), 72.5 °C (reelin, Region 2), 74 °C (BDNF, CpG island 1) or 60 °C (BDNF, CpG island 2) for 15 sec. Detection of the fluorescent products occurred at the end of the 15 sec temperature step. For a melting curve analysis, PCR products were melted by increasing the temperature in 1 °C increments beginning at 60 °C. 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. Detection of methylated DNA was performed using primers complementary to Region 1 of reelin and CpG Islands 1 and 2 of BDNF listed above. Sequences for primers to the reelin promoter were: forward, 5'-GTAGTCGGCGTAGATAAAAGAATAGC-3' and reverse, 5'-CCGAAAAACAAAAACTACTCGAC-3'. Primer sequences to detect methylated DNA in the BDNF1 promoter were: CpG island 1: forward, 5'-
GTACGCTTTGCGGAGAAATCG-3' and reverse, 5'-CAACCTATAGCGAATCTGAATC-3'; CpG island 2: forward, 5'-ATCGGGAAATTTTGAAAGTGCACTG-3' and reverse, 5'-GCAAACGAAAACAAAAACCGAA-3'.

Reactions were performed as described above, with the final step at 75.5 °C (BDNF, CpG island 1) or 60 °C (BDNF, CpG island 2) for 15 sec. Due to low levels of methylation at the region of the reelin promoter, a “kick-start” method was used to ensure detection of a product. First, a conventional PCR reaction was performed in a total volume of 20 µl, consisting of [µL]: 6 bisulfite modified DNA, 10 iQ SYBR Green Supermix, 1 primer (18 µM), 3 DEPC-treated ddH2O. Reactions were carried out in a PTC-200 Peltier Thermal Cycle (Bio-Rad, Hercules, CA) with the following cycling conditions: 95 °C for 3 min, 20 cycles of 95 °C for 15 sec, 60 °C for 1 min, 72 °C for 1 min, and a final 10 min cycle at 72 °C. The product from the conventional PCR was then further amplified with the same primer sequence and similar cycling conditions as described above with the following changes: 50 cycles instead of 20, and the final step occurred at 79 °C. Likewise, a melting curve analysis and gel electrophoresis was carried out to verify specificity of the final product. For every QPCR reaction, samples were assayed in triplicate, and the Ct for each sample was chosen within the linear range. Samples were normalized to β-tubulin-4 (forward, 5'-GGAGAGTAATATGAATGATTTTG-3' and reverse, 5'-CATCTCTCACTTTCCCTAATACCTAATGTTG-3'), and the comparative Ct method was used to calculate differences in gene expression between samples (38,39).

**Histone Extraction.** Hippocampal tissue was homogenized with a Dounce homogenizer using 6 strokes in 1 ml of ice-cold homogenizing buffer (in mM: 250 sucrose, 50 Tris, pH 7.5, 25 KCl, 0.5 phenylmethylsulfonyl fluoride, 1% protease inhibitor mixture (Sigma), 0.9 Na+-butyrate). Tissue homogenates were centrifuged at 7,700 X g for 1 min. The supernatant was taken as the cytoplasmic fraction. Pelleted nuclei were resuspended in 250 µl of 0.4 N H2SO4, incubated on ice for 30 min, and then centrifuged at 4 °C again for 10 min at 14,000 X g. The supernatant was transferred to a fresh tube, and proteins were precipitated with 125 µl of 100% trichloroacetic acid containing 4 mg/ml deoxycholic acid (Na+ salt, Sigma) by incubation on ice for 30 min. The supernatant was discarded, and the protein pellet was washed with 1 ml of ice-cold acidified acetone (0.1% HCl) followed by 1 ml of ice-cold acetone for 5 min each. Protein precipitates were collected between washes by centrifugation at 14,000 X g for 5 min. The resulting purified proteins were resuspended in 10 mM Tris (pH 8.0) and stored at -80 °C. Protein concentrations were determined using the Bio-Rad protein assay reagent.

**Western blotting.** Protein extracts (1 µg) were separated by SDS-PAGE on a 15% resolving gel with a 4% stacking gel and transferred onto PVDF membrane. After transfer, the PVDF membranes were briefly rinsed with 100% methanol, air-dried for 15 min, and washed with 25 ml TTBS (in mM: 0.1% Tween 20, 50 Tris-HCl, pH 7.5, 150 NaCl) for 5 min at room temperature. The membranes were then incubated overnight at 4 °C with an antiserum against proteins of interest in primary buffer (in mM: 0.1% Tween 20, 50 Tris-HCl, pH 7.5, 150 NaCl, and 5% bovine serum albumin). This was followed by three washes in TTBS and incubation for 2 hours with an HRP-conjugated secondary anti-rabbit antibody in TTBS (1:10,000) at RT. The membranes were washed in TTBS and blots were developed using enhanced...
chemiluminescence (ECL) and exposed to film (BioMax, Kodak). Antibodies—The anti-rabbit primary antibodies used, and their dilutions were as follows: anti-Histone H3 (1:1,000), anti-acetyl Histone H3 (Lys-14, 1:1,000), anti-Histone H4 (1:1,000), and anti-acetyl Histone H4 (Lys-5/Lys-8/Lys-12/Lys-16, 1:1000). All antibodies were obtained from Upstate Biotechnology.

Slice electrophysiology. Electrophysiology was performed in an interface chamber (Fine Science Tools, Foster City, CA). Oxygenated ACSF (95%/5% O2/CO2, 30°C) was perfused into the recording chamber at a rate of 1 mL/min. Electrical signals were amplified (A-M Systems, Model 1800, Sequim, WA), digitized (Digidata 1320A, Axon Instruments, Union City, CA) and stored on a PC (Clampex, Axon Instruments). Extracellular stimuli were delivered (Model 2200 Stimulus Isolator, A-M Systems) by placing isonel enamel coated, bipolar platinum-tungsten (92%/8%, 0.0011 in-dia) electrodes on the border of Area CA3 and CA1 along the Schaffer-collaterals. fEPSPs were recorded in stratum radiatum with an ACSF-filled glass recording electrode (1-3 MΩ). All analyses of electrophysiologic traces were performed using Clampfit (Axon Instruments). The relationship between the left slope of the fiber volley (presynaptic depolarization) and the left slope of the fEPSP (postsynaptic depolarization) over various stimulus intensities was used to assess baseline synaptic transmission. All subsequent experimental stimuli were set to an intensity that evoked a fEPSP that had a left slope of 50% of the maximum fEPSP left slope. NMDA-receptor-mediated synaptic transmission was assessed as per baseline synaptic transmission above in the presence of the AMPA-R antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) and a modified ACSF (same as above except: 0 mM MgCl2, 4 mM CaCl2). These conditions allow unblocking of the NMDA-R and allow assessment of NMDA-R function by measuring baseline fEPSPs mediated by this receptor. Paired-pulse facilitation was investigated at various interstimulus intervals (20, 50, 100, 200, 300 msec). Long-term potentiation (LTP) was induced by administering 3 trains of theta-burst stimulation. Each train consisted of 10 sets of bursts (4 stimuli, 100 Hz) with an interburst interval of 200 msec. There was 20 sec between each stimulus train. Synaptic efficacy was monitored 30 min prior to and 180 min following induction by recording fEPSPs every 20 sec (traces were averaged for every 2 min interval). Drug or vehicle was administered to slices for the duration of the experiment beginning 20 min prior to LTP induction. Slices that did not exhibit stable fEPSP slopes during the first 10 min of recording were excluded from the study.

Drugs. Zebularine and 5-Aza-2-deoxyctydine were purchased from CalBioChem (San Diego, CA). CNQX was purchased from Tocris Cookson, Inc. (Ellisville, MO). PDA was purchased from Sigma (St. Louis, MO).

Statistical analysis. One sample t-tests were used to assess changes in the methylation state of regions 1 and 2 of the reelin and BDNF1 promoters, and changes in expression of DNMT genes in the hippocampus. One-way analysis of variance (ANOVA) combined with Bonferroni's multiple comparison test was used to analyze changes in unmethylated DNA between regions 1 and 2 of the reelin promoter. Data relating fEPSP slope to fiber volley slope was fit with a single exponential function (Y = TOP x [1 - e^-K x X]); TOP and K were compared between treatment groups using an F test. PPF and LTP experiments were analyzed using a two-way ANOVA with repeated measures. Analysis of histone acetylation was performed using ANOVA; post-hoc comparisons were made using the Tukey-Kramer test. Significance was set at p ≤ 0.05 for all tests.

RESULTS

We hypothesized that (cytosine-5) DNA methylation in the CNS is, at least in part, a dynamic process that plays a role in information storage. Therefore, we investigated whether genes in the hippocampus could undergo acute changes in methylation status in response to inhibition of DNMT. Reelin is a large (~340 kD) extracellular protein that was first discovered in the context of neural development, but has since been implicated in formation of long-term memory, synaptic plasticity and cognition (40-42). Furthermore, there is some evidence suggesting that the reelin promoter may undergo acute regulation of DNA (cytosine-5) methylation in neural progenitor cells (37). To determine whether DNA (cytosine-5)
methylation was acutely regulated in the adult CNS, we measured DNA (cytosine-5) methylation at 2 different sites within a CpG island present within the reelin promoter (Fig. 1A, see also ref 37). Treatment of hippocampal slices with the DNMT inhibitor Zebularine (Zeb, 100 µM, 40 min) induced a significant and robust increase in unmethylated DNA, and a concomitant decrease in methylated DNA within 1 distinct portion of the CpG island (Fig. 1B, Region 1: -641 bp – -478 bp from transcription start site). A second, distinct site within the CpG island exhibited no change in levels of unmethylated DNA (Fig. 1B, Region 2: -972 bp – -862 bp from transcription start site), indicating that treatment with Zeb resulted in region-specific changes in DNA methylation. To further investigate regulation of DNA methylation in the adult brain, methylation of DNA was measured in two CpG islands detected in silico (Fig. 2A) in the promoter of Exon 1 in BDNF. Treatment with Zeb induced a significant increase in unmethylated DNA within CpG island 1 (Fig. 2B, CpG island 1: -603 bp to – -412 bp from transcription start site). A second CpG island, distal from exon 1, exhibited no change in unmethylated or methylated DNA in response to treatment with Zeb (Fig. 2C, CpG island 2: -1170 bp – -1018 bp from transcription start site). These results suggest that specific regions within the reelin and BDNF promoter can undergo acute changes in DNA (cytosine-5) methylation status in the adult hippocampus, and suggest that acute modulation of DNA (cytosine-5) methylation occurs in the adult brain.

Activation of PKC is an important step in induction of synaptic plasticity in the hippocampus (43). Hippocampal slices were treated with phorbol-12,13-diacetate (PDA, 3 µM, 20 min) to assess acute regulation of DNA (cytosine-5) methylation in Area CA1 in response to activation of PKC. In the region of the reelin promoter that exhibited rapid changes in DNA methylation after treatment with Zeb, PDA induced a significant decrease in methylated DNA with a corresponding trend for an increase in unmethylated DNA (Fig. 3A, Region 1, t = 14.3, df = 3, p < 0.001). Moreover, PDA had no effect on the region of the reelin promoter that showed no regulation in response to Zeb (Fig. 3A, Region 2). These results indicate activation of PKC in Area CA1 of the hippocampus results in rapid demethylation of a specific region of the reelin promoter.

The family of DNMT enzymes mediates methylation of DNA (1-5). The DNMT isoforms 1, 3A and 3B are expressed in the adult mammalian CNS (13,44,45). Several studies suggest that the ras-MEK-ERK signaling pathway, a pathway essential for induction of synaptic plasticity and formation of long-term memory (36), regulates expression of DNMT genes in nonneuronal cells (46-48). PKC is one of many signaling molecules that have been implicated in the induction of synaptic plasticity and memory formation in the hippocampus through activation of the ras-MEK-ERK signaling cascade (43). If regulation of DNA (cytosine-5) methylation is involved in synaptic plasticity, then it is possible that regulation of DNMT expression occurs in response to activation of signaling pathways responsible for induction of synaptic plasticity. We determined whether DNMT gene expression was regulated in response to activation of PKC. Expression of DNMT3A was significantly increased in Area CA1 of the hippocampus in response to treatment with phorbol-12,13-diacetate (Fig. 3B, PDA, 3 µM, 45 min, t = 3.1, df = 5, p < 0.05). Expression of DNMT1 and DNMT3B was unaffected by PDA (Fig. 3B, DNMT1: t = 1.3, df = 5, p < 0.3; DNMT3B: t = 1.5, df = 5, p < 0.2). The immediate early gene c-fos was also significantly increased by PDA (Fig. 3B, t = 4.5, df = 7, p < 0.005). These results suggest that the expression of DNMT3A in the hippocampus is acutely responsive to the PKC signaling cascade.

Thus far, we have observed that DNA (cytosine-5) methylation can undergo acute regulation and that expression of DNMT3 mRNA is regulated by the PKC signaling cascade, which is activated during induction of LTP (49-51). These results suggest that DNMT activity could play a role in modulating neural function, especially during induction of synaptic plasticity. To assess the potential role that DNMT activity might play in regulating the induction of synaptic plasticity, we determined the effect of two distinct inhibitors of DNMT on theta-burst stimulus-induced LTP. Theta-burst stimulation resulted in induction of robust LTP that persisted for at least 3 h (Figs. 4A,B). Exposure of slices to either Zeb (Fig. 4A,
100 µM) or 5-Aza-2-deoxycytidine (5-Aza, Fig. 4B, 30 µM) resulted in an immediate and significant diminution of LTP that persisted for up to 3 h after induction (Zeb: $F_{[1,20]} = 451$, $p < 0.0001$, animals: $n = 4$, slices: $n_{\text{DMSO}} = 11$, $n_{\text{Zeb}} = 10$; 5-Aza: $F_{[1,49]} = 283$, $p < 0.0001$, animals: $n = 9$, slices: $n_{\text{CH3COOH}} = 24$, $n_{5\text{-Aza}} = 26$). To determine whether inhibition of DNMT had any effect on basal synaptic efficacy, slices were exposed to either Zeb or 5-Aza for 3.3 h and synaptic efficacy was monitored. Neither Zeb nor 5-Aza had significant effects on basal synaptic efficacy relative to slices treated with the respective vehicle alone (Figs. 4C,D, respectively), suggesting that their effects on LTP were not due simply to depression of baseline transmission (Zeb: $F_{[1,35]} = 0.6$, $p < 1.0$, animals: $n = 6$, slices: $n_{\text{DMSO}} = 10$, $n_{\text{Zeb}} = 26$; 5-Aza: $F_{[1,47]} = 0.8$, $p < 1.0$, animals: $n = 7$, slices: $n_{\text{CH3COOH}} = 25$, $n_{5\text{-Aza}} = 23$).

To further investigate the possible effects of DNMT inhibition on induction of LTP, several aspects of basal synaptic transmission were measured after 20 min applications of either Zeb or 5-Aza, the time at which LTP was induced in the experiments described above. Treatment of slices with either Zeb (Fig. 5A) or 5-Aza (Fig. 5B) had no significant effect on the input-output function for basal synaptic transmission (Zeb: $F_{[2,18]} = 2.5$, $p < 0.2$, animals: $n = 4$, slices: $n_{\text{DMSO}} = 14$, $n_{\text{Zeb}} = 15$; 5-Aza: $F_{[2,20]} = 0.2$, $p < 0.9$, animals: $n = 3$, slices: $n_{\text{CH3COOH}} = 10$, $n_{5\text{-Aza}} = 11$). Short-term plasticity of neurotransmitter release, as assessed by paired-pulse facilitation, was also normal in slices treated with either Zeb (Fig. 5C, $F_{[1,26]} = 1.9$, $p < 0.2$, animals: $n = 4$, slices: $n_{\text{DMSO}} = 12$, $n_{\text{Zeb}} = 15$) or 5-Aza (Fig. 5D, $F_{[1,18]} = 0.5$, $p < 0.5$, animals: $n = 3$, slices: $n_{\text{CH3COOH}} = 9$, $n_{5\text{-Aza}} = 11$).

One explanation for the effects of DNMT inhibitors on LTP is that the drugs might non-specifically block NMDA receptors, the function of which is required for theta-burst LTP at Schaffer collateral synapses. To test this possibility we monitored the effect of Zeb and 5-Aza on NMDA receptor-mediated fEPSPs in our slice preparation. Zeb had no effect on NMDA-receptor-mediated synaptic responses (Fig. 5E, $F_{[2,14]} = 0.3$, $p < 0.8$, animals: $n = 4$, slices: $n_{\text{DMSO}} = 9$, $n_{\text{Zeb}} = 15$). However, NMDA-receptor-mediated synaptic transmission was enhanced in 5-Aza-treated slices relative to slices exposed to the vehicle (0.001% CH$_3$COOH, Fig. 5F, $F_{[2,9]} = 13$, $p < 0.005$, animals: $n = 3$, slices: $n_{\text{CH3COOH}} = 9$, $n_{5\text{-Aza}} = 10$). These results indicate that neither Zeb nor 5-Aza has a deleterious effect on NMDA-R function.

Another explanation for how DNMT inhibitors block induction of LTP is that they inhibit the postsynaptic response to the theta-burst stimulation, either through direct interactions with ion channels or indirectly through ERK stimulation (see 52). Therefore, theta-burst responses represent a physiologic measure of the efficacy of LTP induction. Theta-burst responses were not affected by Zeb (Fig. 6A,B,E, $F_{[1,18]} = 0.1$, $p < 0.8$) or 5-Aza (Fig. 6C,D,F, $F_{[1,57]} = 1$, $p < 0.4$). Considered together, all of these results indicate that neither Zeb nor 5-Aza had any deleterious effect on synaptic transmission, short-term plasticity, NMDA receptor function, or induction of LTP, suggesting that the immediate diminution of LTP by inhibitors of DNMT-activity is not due to an indirect effect on collective processes required for LTP induction.

In a previous study, we reported that levels of histone H3 acetylation were increased in response to activation of NMDA-receptors and the PKC signaling cascade (23). Moreover, increasing levels of histone acetylation with histone deacetylase inhibitors enhanced induction of LTP (23). Levels of DNA methylation can affect levels of histone acetylation (28-32), suggesting that the blockade of LTP induction by DNMT inhibitors could be due to modulation of histone acetylation. Many studies indicate that increases in histone acetylation are necessary for successful induction of LTP in the hippocampus (23,25-27). Therefore, we hypothesized that DNMT inhibition prevented increases in histone acetylation necessary for induction of LTP. To test this hypothesis, we measured PKC-mediated increases in histone H3 acetylation in the presence or absence of Zeb or 5-Aza. As previously reported, treatment of hippocampal slices with PDA (3 µM, 20 min) significantly increased acetylation of histone H3 (Fig. 7A,B, $p < 0.05$) but not histone H4 (Fig. 7C,D, $p > 0.05$). Pretreatment of slices with Zeb (100 µM, 40 min) or 5-Aza (30 µM, 40 min) completely inhibited the PDA-induced increase in histone H3 acetylation (Fig. 7A,B). Neither Zeb
DISCUSSION

DNA methylation represents one of the most ancient mechanisms of cellular memory. Once differentiated, every cell in a metazoan must “remember” its appropriate pattern of gene expression for the organism to survive and function normally. Studies of developmental biology have established a role for DNA methylation in triggering this differentiated state of the cell. Traditionally, DNA methylation is thought of as a static process; once a cell becomes differentiated and its genome appropriately methylated, no further changes in methylation status of the genome occurs. In support of this “DNMT-stasis” hypothesis, the activity and expression of DNMT significantly decreases in differentiated cells, and is positively correlated with the proliferative state of cells. The brain, however, represents the greatest exception to this rule. The mature brain is made up almost exclusively of terminally differentiated non-dividing cells. But surprisingly, high levels of DNMT mRNA persist even into adulthood.

Our study provides evidence that DNMT plays a role in modulating adult nervous system function. First, we have shown that rapid changes occur in the DNA (cytosine-5) methylation status of 2 genes implicated in synaptic plasticity in the adult hippocampus. We provide evidence that DNMT activity plays a role in the modulation of plasticity induction in the hippocampus. We demonstrated that engagement of the PKC signaling cascade, a cascade involved in induction of synaptic plasticity and long-term memory formation, decreases methylation of reelin and increases expression of DNMT3A mRNA. Finally, we show that inhibition of DNMT activity can block PKC-mediated changes in histone acetylation. All of these results suggest that DNMT gene expression is actively regulated, and that DNMT activity can have dramatic and rapid effects on DNA methylation and induction of plasticity in the hippocampus.

Several recent reports indicate that the brain has co-opted one form of epigenetic tag, histone acetylation, to subserve induction of long-term synaptic plasticity, formation of long-term memory, and cognitive function in general (for review, see 24). The results presented here suggest that another form of epigenetic tag, direct covalent modification of DNA, has been co-opted by the brain for use in regulation of the induction of lasting synaptic plasticity. Our results suggest that in at least some regions of chromatin, DNA (cytosine-5) methylation is a dynamic signal that is modulated to regulate the functional state of the neuron. Earlier studies of DNA (cytosine-5) methylation observed depolarization-induced decreases in methylation of BDNF at syntenic regions in rat (promoter 3) and mouse (promoter 4) embryonic cortical neurons (33,34). In this regard, our results are the first to suggest a link between the direct, covalent modification of DNA and the regulation of synaptic plasticity in the fully developed, adult nervous system (see also 54,55,56).

Our studies have several caveats to their interpretation. The first concerns the specificity of the DNMT inhibitors. While we used two structurally distinct DNMT inhibitors, and assessed a number of control parameters for synaptic function, it is possible that the inhibitors are acting at a site distinct from blockade of DNA methylation. Even if the inhibitors are acting specifically to block DNA methylation, a second possibility must be considered. We do not know if the inhibitors are blocking an activity-dependent signaling process necessary for LTP induction or if they are disrupting a DNA methylation state that determines a set-point controlling the likelihood of triggering lasting synaptic plasticity. Therefore, we are currently unable to distinguish between a role for DNMTs as a component of a signal transduction mechanism versus DNMTs as a component of an ongoing mechanism maintaining the neuron in a functional state capable of synaptic potentiation. We should note however, that no deleterious effects were observed in any measure

nor 5-Aza alone had any effect on acetylation of either histone H3 (Fig. 7A,B, p > 0.05) or histone H4 (Fig. 7C,D, p > 0.05). These findings confirm our earlier report of PDA-induced increases in H3 acetylation in vitro, and suggest a role for DNMT activity in the metaplasticity of histone H3 acetylation (53). Moreover, these findings further support the hypothesis that dynamic changes in DNA (cytosine-5) methylation specifically and modulation of the epigenome in general plays a role in information storage in the CNS.
There are several different temporal phases of synaptic plasticity, each with their own unique requirements for induction. The early phases of LTP (E-LTP), for example, require posttranslational modification of existing proteins, but do not require macromolecular synthesis. However, inhibition of DNMT activity has a robust effect on E-LTP. Even though E-LTP does not require transcription, treatments that modulate overall transcriptional state have been shown to influence E-LTP. For example, inhibition of histone deacetylase results in a robust and rapid transcription-dependent enhancement of E-LTP (23). Thus, while E-LTP does not require transcription for its induction, modulators of transcription that act through alteration of the epigenetic state of a cell can affect the capacity for induction and/or expression of E-LTP.

DNA (cytosine-5) methylation has been universally associated with down-regulation of gene expression (57). Methylation of DNA can interfere with the ability of transcription factors to bind to cis-regulatory elements within the DNA, and can attract methyl-CpG binding proteins, which mediate gene repression through the recruitment of chromatin remodeling enzymes such as histone deacetylases (28-32). This presents a conundrum relative to the results we have presented, as formation of long-term forms of synaptic plasticity and memory have been historically associated with increases in gene expression. Thus, one might expect that inhibition of DNMT might actually increase overall levels of transcription and actually potentiate synaptic plasticity and/or memory formation. There is evidence that repression of gene expression might be as important as upregulation of gene expression during formation of lasting synaptic plasticity (58,59). Thus, dynamic DNA methylation might play a role in repression of genes that act to inhibit formation of long-term plasticity. Furthermore, once established, DNA methylation may also act to stabilize potentiated neurons through downregulation of the processes used to induce a plastic state.

In conclusion, our results are the first to suggest that DNA (cytosine-5) methylation in adult brain is, in part, a dynamic process involved in signaling and possibly encoding the functional state of the neuron. These results are striking, as they are the first to suggest that DNA in non-proliferating neurons is directly covalently modified as a mechanism for regulating synaptic strength. These results further support the notion that a “cellular memory” in the form of epigenetic marking of chromatin has been co-opted by the nervous system to serve in cellular processes requiring plasticity, and for memory-related cognition in general.

REFERENCES

FOOTNOTES
This work was supported by MH57014 (J.D.S.).

FIGURE LEGENDS

**Figure 1.** Dynamic regulation of DNA methylation in the *reelin* promoter in the CNS. **A)** Schematic representation of the location of the CpG island in the Reelin promoter region relative to the transcription initiation site. Primer sets as described in the methods were designed to amplify regions 1 or 2. **B)** Upper panel: Quantitative real-time PCR was used to determine the fold change in methylated or unmethylated DNA in zebularine-treated CA1 slices relative to control treatment. *, p < 0.05, n=5, One sample t test. ***, p < 0.05, n=5, analysis of variance with Bonferroni’s multiple comparison test. Vertical bars represent SEM. Lower panel: Representative agarose gel electrophoresis showing increased unmethylated DNA and decreased methylated DNA (specific to Region 1) in zebularine-treated samples (Z) relative to control samples (C). *, molecular weight marker, upper 200bp, lower 100bp.

**Figure 2.** Dynamic regulation of DNA methylation in the *BDNF* promoter in the CNS. **A)** Schematic representation of the location of CpG islands in the promoter region upstream of exon 1 in the BDNF gene. Primer sets as described in the methods were designed to amplify islands 1 or 2. **B)** Quantitative real-time PCR was used to determine the fold change in methylated or unmethylated DNA in zebularine-treated CA1 slices relative to control treatment. Treatment with Zebularine significantly increased
unmethylated DNA and had no effect on methylated DNA within CpG island 1. C) Zebularine had no effect on methylated or unmethylated DNA in CpG island 2. *, p < 0.05, n=4 - 5, One sample t test. Vertical bars represent SEM. Representative agarose gel electrophoresis, to the right of summary data, showing increased unmethylated DNA (CpG island 1) in zebularine-treated samples (Z) relative to control samples (C). *, molecular weight marker, upper 200bp, lower 100bp.

**Figure 3.** Methylation of the reelin promoter is decreased and expression of DNMT3 is increased in response to phorbol ester stimulation. Transverse hippocampal slices were exposed to phorbol-12,13-diacetate (PDA, 3 μM). Tissue was processed immediately after treatment. A) Methylation of the reelin promoter in Region 1 (see Fig. 1A) is significantly decreased by PDA (20 min) relative to DMSO-treated controls. B) Expression of DNMT isoforms (1, 3A, 3B) was measured using real-time, RT-QPCR. Treatment with PDA (45 min) significantly increased expression of the immediate-early gene c-fos, which was used a positive control. Expression of DNMT1 and DNMT3B was unaffected by PDA treatment. Expression of DNMT3A was increased by PDA. Asterisk (*) indicates significant difference (P < 0.05) from vehicle-treated control tissue as determined by One-Sample t-test. Error bars indicate SEM.

**Figure 4.** Inhibitors of DNMT block LTP. Transverse hippocampal slices were exposed to either a DNMT inhibitor or vehicle (grey bar), and synaptic efficacy was monitored. A) LTP induced with theta-burst stimulation was diminished in slices treated with Zebularine (100 μM, open circles) relative to slices treated with vehicle (0.5% DMSO, closed boxes). Representative traces are shown 2 min before (grey line) and 180 min after (black line) LTP induction. Calibration bar indicates 1 mV and 5 msec. B) LTP induced with theta-burst stimulation was diminished in slices treated with 5-Aza-2'-deoxycytidine (30 μM, open circles) relative to slices treated with vehicle (0.001% CH₃COOH, closed boxes). Representative traces are shown 2 min before (grey line) and 180 min after (black line) LTP induction. Calibration bar indicates 2 mV and 5 msec. C) Synaptic efficacy was not affected by Zebularine (100 μM) in the absence of LTP induction. Representative traces are shown 18 min (grey line) and 198 min after (black line) initial exposure to Zebularine or vehicle. Calibration bar indicates 2 mV and 5 msec. D) Synaptic efficacy was not affected by 5-Aza-2'-deoxycytidine (30 μM) in the absence of LTP induction. Representative traces are shown 18 min (grey line) and 198 min after (black line) initial exposure to 5-Aza or vehicle. Calibration bar indicates 2 mV and 5 msec. In all panels, error bars indicate SEM.

**Figure 5.** Inhibitors of DNMT do not affect synaptic transmission. Transverse hippocampal slices were exposed to a DNMT inhibitor or vehicle for 20 min, and Schaffer-collateral synaptic transmission was assessed. A) Zebularine (100 μM) had no significant effect on synaptic transmission relative to vehicle (0.5% DMSO) controls. B) 5-Aza-2'-deoxycytidine (30 μM) had no significant effect on synaptic transmission relative to vehicle (0.001% CH₃COOH) controls. C) Zebularine had no effect on paired-pulse facilitation relative to vehicle-treated controls. Data are offset +/- 2.5 msec for visualization. D) 5-Aza had no effect on paired-pulse facilitation relative to vehicle-treated controls. Data are offset +/- 2.5 msec for visualization. E) Zebularine had no effect on NMDA-receptor-mediated synaptic transmission. F) NMDA-receptor mediated synaptic transmission was normal in 5-Aza treated slices, but depressed in vehicle-treated slices, indicating that CH₃COOH causes a modest diminution in NMDA-receptor function. In all panels, error bars indicate SEM.

**Figure 6.** Inhibitors of DNMT do not affect theta-burst-induced depolarization. Depolarization during theta-burst stimulation was measured in the presence of vehicle or DNMT inhibitor. Theta-burst stimulation consists of 10 bursts administered at 5 Hz. Each burst consists of 4 stimuli delivered at 100 Hz. A) Representative traces showing theta bursts in a slice treated with DMSO (0.5%), the vehicle for Zeb. B) Representative traces showing theta bursts in a slice treated with Zeb (100 μM). C) Representative traces showing theta bursts in a slice treated with CH₃COOH (0.001%), the vehicle for 5-Aza. D) Representative traces showing theta bursts in a slice treated with 5-Aza (30 μM). E) Summary quantification of area under the curve for each theta burst of slices treated with DMSO and Zeb. No significant differences were observed. F) Summary quantification of area under the curve for each theta...
burst of slices treated with CH₃COOH and 5-Aza. No significant differences were observed. Scale bars indicate 20 msec and 5 mV. Error bars are SEM.

**Figure 7.** Regulation of histone H3 and H4 acetylation by DNMT. Transverse hippocampal slices were exposed to vehicle (Veh), DNMT inhibitor (Zeb or 5-Aza), PDA or DNMT inhibitor followed by PDA and levels of H3 and H4 acetylation were assessed using Western blot analysis. **A)** Levels of histone H3 acetylation were significantly changed by our experimental treatment ($F_{[3,12]} = 5.10, p < 0.05$). Post-hoc analyses revealed that PDA treatment significantly increased levels of acetylated histone H3 relative to veh-treated control tissue from area CA1 ($p < 0.05$). Pretreatment with Zeb blocked the PDA-induced increase in H3 acetylation ($p > 0.05$). Treatment with Zeb alone had no effect on H3 acetylation ($p > 0.05$). **B)** Levels of histone H3 acetylation were significantly changed by our experimental treatment ($F_{[3,12]} = 29.42, p < 0.05$). Post-hoc analyses revealed that PDA treatment significantly increased levels of acetylated histone H3 relative to veh-treated control tissue from area CA1 ($p < 0.05$). Pretreatment with 5-Aza blocked the PDA-induced increase in H3 acetylation ($p > 0.05$). Treatment with 5-Aza alone had no effect on H3 acetylation ($p > 0.05$). **C)** Acetylation of histone H4 was unaffected by PDA or Zeb ($F_{[3,8]} = 3.62, p > 0.05$). **D)** Acetylation of histone H4 was unaffected by PDA or 5-Aza ($F_{[3,12]} = 0.79, p > 0.05$). Asterisk (*) indicates significant difference from Veh as determined by post-hoc Tukey-Kramer test. Error bars indicate SEM.
Figure 1

A

CpG Island

Reelin

-2300 -1000 start 100 700 bp

Region 2  Region 1

B

Fold change relative to control

6

5

4

3

2

1

0

Unmethylated

Methylated

Unmethylated

Region 1

Region 2

Unmethylated  Tubulin  Methylated  Tubulin

Unmethylated  Tubulin

Region 1  Region 2
Figure 2

A

CpG Island 2

BDNF

- 1223

CpG Island 1

- 223 bp

Exon 1

start

B

CpG Island 1

Fold change relative to control

Unmethylated

Methylated

Unmethylated

Methylated

Unmethylated

Methylated

C

C

C

C

Z

Z

Z

Z

Unmethylated

Methylated

Tubulin

Tubulin

Tubulin

Tubulin

C

C

C

C

Z

Z

Z

Z

*
Figure 3

A

DNA Methylation (Fold Change)

Unmethylated  Methylated  Unmethylated

Region 1  Region 2

B

mRNA Expression (Percent Control)

DNMT1  DNMT3A  DNMT3B  c-Fos

*
A DMSO (0.5%)

B Zebularine (100 μM)

C CH₃COOH (0.001%)

D 5-Aza-2-deoxycytidine (30 μM)

E

F
Figure 7
Evidence that DNA (Cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus
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