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RESEARCH ARTICLE

Dynamic methylation driven by neuronal activity in hippocampal neurons impacts complex behavior

Anita E. Autry, Megumi Adachi, Lisa M. Monteggia (✉)

Department of Neuroscience, The University of Texas Southwestern Medical Center, Dallas, TX 75390-9070, USA

Abstract **Epigenetic processes are well-known to play critical roles in learning and memory. Among epigenetic processes, accumulating data suggests that DNA methylation in particular is a critical determinant of learning and memory. *In vitro* data have suggested that DNA methyltransferase inhibitors can trigger DNA demethylation and subsequent gene expression of the brain-derived neurotrophic factor gene in an activity dependent manner. To examine if these processes occur *in vivo*, we chronically infused DNMT inhibitors into the hippocampus and examined the impact on behavior. We find that chronic DNMT inhibition in the hippocampus results in increased anxiety-related behavior and deficits in context-dependent fear conditioning accompanied by an increase in BDNF expression. Gene expression changes were blocked by pretreatment with the NMDA receptor antagonist AP5, suggesting that DNMT inhibition enhances gene expression in an activity-dependent manner and that, conversely, the behavior deficits and abnormal gene expression are facilitated by NMDA receptor activity.**

Keywords DNA methylation, NMDA receptors, learning and memory, behavior, hippocampus

Introduction

Epigenetic processes are critical modulators of gene transcription in the nervous system. During development, epigenetic mechanisms are important for establishing the functional identity of cell types. However, recent data have shown that alterations in epigenetic processes in the adult central nervous system can impact gene expression and thereby mediate complex processes such as learning and memory (Levenson and Sweatt, 2006; Nelson and Monteggia, 2011; Rudenko and Tsai, 2014; Zovkic et al., 2014). DNA methylation is an example of an epigenetic process that plays an essential role in the repression of gene expression. The significance of DNA methylation in the brain is becoming more apparent by its association with neurological and psychiatric disorders. For instance, Rett Syndrome is a neurodevelopmental disorder caused by mutations in the methyl-CpG binding protein 2 (*MECP2*) gene that is crucial for interpreting DNA methylation and controlling gene expression (Amir et al., 1999). Fragile X and ICF (immunodeficiency, centromeric region instability, facial anomalies) syndromes, on the other hand, arise from dysregulation in normal DNA methylation (Hansen et al., 1999; Turner et al., 1996). More recent data have implicated a role of DNA methylation in learning and memory and in disorders including depression, bipolar disorder and schizophrenia (Li et al., 2013; Morris and Monteggia, 2014; Peña et al., 2014; Day et al., 2015).

DNA methyltransferases (DNMTs) are enzymes that transfer methyl groups onto cytosine residues in DNA, regulating *de novo* methylation or maintenance of methylation and compacting local chromatin structure and thus gene silencing (Bird, 2002). Methylcytosine analogs such as 5-azacytidine (5azaC) and zebularine (Zeb) have been used to examine the role of DNMTs in neurons. Acute infusion of DNMT inhibitors into the hippocampus impairs long-term potentiation and contextual fear conditioning, a hippocampal dependent learning task (Levenson and Sweatt, 2006; Miller and Sweatt, 2007). *In vitro* studies have demonstrated that prolonged depolarization of cortical neurons results in demethylation of brain-derived neurotrophic factor (BDNF), a neurotrophin linked to synaptic plasticity (Martinowich et al., 2003). In previous work, our laboratory demonstrated that

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Correspondence: Lisa M. Monteggia
E-mail: lisa.monteggia@utsouthwestern.edu

chronic application of 5azaC or Zeb in postnatal hippocampal cultured neurons selectively decreases miniature excitatory postsynaptic potentials (mEPSCs), which impacts neuronal excitability and network activity (Nelson et al., 2008). We were also able to demonstrate that chronic DNMT inhibitor treatment produced an activity dependent demethylation of the BDNF gene in hippocampal neurons, resulting in increased BDNF expression. This increase was mediated by synaptic activation of NMDA receptors as shown by the ability of the NMDA receptor antagonist, AP5, to block the effects and thus demonstrating a mechanistic link between changes in DNA methylation and synaptic function.

The goal of this study was to extend our previous *in vitro* data by examining if chronic DNMT inhibitor infusion into the hippocampus *in vivo* produces behavioral phenotypes that could be rescued by the NMDA receptor antagonist, AP5. To address this goal, we chronically infused 5azaC or Zeb into the dentate gyrus sub-region of the hippocampus in mice and examined them in an array of behavioral paradigms. We then observed if AP5 could rescue the DNMT mediated behavioral phenotypes. Collectively, these experiments showed that NMDA receptor mediated synaptic activity impacts behavioral phenotypes sensitive to DNMT inhibition *in vivo*.

Materials and methods

Mice

C57BL/6 male mice aged 6 – 8 weeks old were habituated to the animal facility for one week prior to surgery. Mice were kept on a 12 h/12 h light dark cycle and given access to food and water *ad libitium*. All experiments were performed and scored by an observer that was blind to the mouse treatment. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

Surgery

Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine in 0.9% saline. Osmotic minipumps (Alzet) containing either vehicle 10% DMSO/saline (n = 12) or Zeb (600 ng/μL; n = 12) or vehicle 0.8% acetate/saline (n = 12) or 5azaC (200 ng/μL; n = 12) were implanted into mice. In subsequent experiments to examine the behavioral reversal by AP5, animals were infused with either vehicle alone 0.8% acetate/saline (n = 10), 5azaC alone (200 ng/μL; n = 10), AP5 alone (0.5 mM; Sigma) (n = 10) or 5azaC (200 ng/μL) + AP5 (0.5 mM) (n = 10). Pumps had a flow rate of 0.25 μL/h over 14 days. Pumps were connected to bilateral cannulae (Plastics One) as previously described (Adachi et al., 2009) and implanted at anteroposterior −2.0 relative to bregma. After surgery mice were singly housed and recovered for five days with daily handling until commencement of behavioral testing. The order of behavioral testing was from least to most stressful (Fig. 1A).

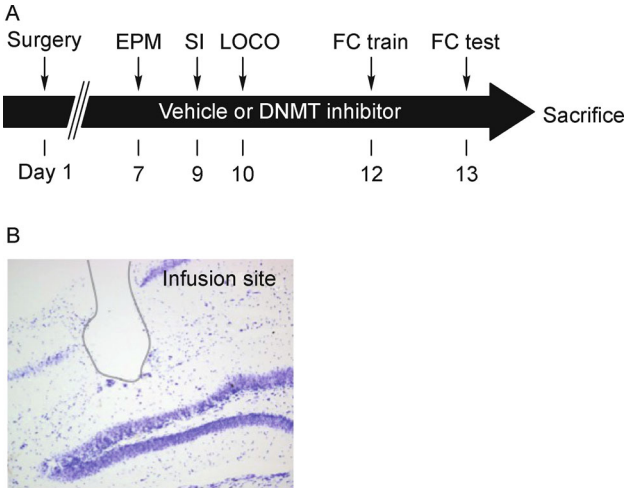

Figure 1 Overview of the infusion paradigm. (A) Mice were implanted with bilateral cannulae directed toward the dentate gyrus attached to a minipump and allowed to recover from surgery for six days. Starting on day 7, mice were tested for behavioral impact of chronic infusion of either vehicle or DNMT inhibitors 5azaC or zebularine (Zeb) including elevated plus maze, social interaction, locomotion, and fear conditioning. (B) Cannula placement was confirmed by examining the location of the cannula track in cressyl violet stained tissue.

Behavior

Elevated plus maze

Mice were placed in the center of a plus maze containing two closed arms and two open arms elevated ~2 feet above the floor in dim lighting (40 lx) as previously described (Na et al., 2014). Exploratory behavior was monitored for 5 min with video tracking and time spent in closed and open arms was quantified by EthoVision software (Noldus Information Technology).

Social interaction

Social interest was measured as approach of an experimental mouse to a novel target male C57BL/6 mouse. An experimental mouse was placed in an open field arena 42 cm^2 with a wire mesh cage against one side and allowed to explore for 5 min with movement tracked by EthoVision software. After the first trial, the test mouse was removed while a target mouse was placed behind the wire mesh and then the experimental mouse reintroduced into the arena for another 5 min. EthoVision Software linked to video tracking measured duration of time spent in the interaction zone in the absence and presence of a target mouse.

Locomotor activity

Mice were placed in a standard cage with fresh bedding and

locomotor activity was recorded for 2 h under red light by photocell beams linked to computer acquisition software (San Diego Instruments).

Fear conditioning

Fear conditioning was assessed as previously described (Monteggia et al., 2004; Na et al., 2012). Briefly, mice were placed in an individual chamber for 2 min followed by a 90 dB tone for 30 s immediately followed by a 0.5 mA footshock for 2 s followed by 1 min of no stimulation after which the tone-shock pairing was repeated. Mice remained in the chambers for 1 min after the second tone and shock before being returned to their home cages. The individual chambers were cleaned between each mouse. To test for context-dependent fear conditioning, the mice were placed back in the same individual chamber 24 h later with no tone or shock and observed for freezing behavior for 5 min by an observer blind to treatment. Freezing behavior was defined as no movement except for respiration.

Placement confirmation

At the end of behavior testing, mice were decapitated and fresh brains were dissected laterally; half the brain was post-fixed in 4% paraformaldehyde for 72 h, processed in 30% sucrose overnight, and sectioned at 30 μM thickness and Nissl stained to verify cannula placement. The other half of the brain was sectioned and punches from dentate gyrus were dissected for RNA isolation.

Quantitative RT-PCR

Tissue punches from dentate gyrus were dissected and total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. Conditions for cDNA synthesis, amplification, and BDNF primer sequences were described previously (Berton et al., 2006; Miller and Sweatt, 2007). Other primer sequences: Reelin, Forward 5′-ATG CAG CAA CTG TGA GAT GGG AGA-3′ and Reverse 5′-TTG TTG TGT TCA GGC ATG TGG-5′, PP1 Forward, 5′-AGA GCC CAT CAG GTG GTT GAA GAT-3′ and Reverse, 5′-CTG CAT TGT CAA ACT CGC CAC AGT-3′. Fold change in BDNF, reelin, and PPI expression was normalized to ribosomal subunit 18S RNA.

Statistical analysis

Locomotor data were analyzed with repeated measures ANOVA using SPSS software. Data comparing control group to experimental group were analyzed with an unpaired 2-tailed Student's *t*-test. All other data were analyzed by a one-way ANOVA followed by post-hoc analysis for multiple comparisons by Dunnett's test or Neuman Keul's correction to assess the differences among groups. Data are presented as mean±SEM and statistical significance was considered $p < 0.05$.

Results

Confirmation of cannulae placements

We used bilateral cannulae targeted to the dentate gyrus to infuse chronically the DNMT inhibitors 5azaC or Zeb into adult male mice to examine their impact on behavior (Fig. 1A). Following surgery, the mice were allowed to recover for 6 days prior to behavioral testing. The order of the behavioral testing was from the least to more stressful paradigms. The mice were sacrificed 14 days after surgery and the placement of the cannulae confirmed in each mouse using Nissl staining to show cannula tracks as well as cell bodies. Our placements were considered on target if they were inside the medial bend of the dentate gyrus (Fig. 1B). All data from animals with 'off target' infusions were removed from analysis (only one surgery from the AP5 group did not meet criteria). Examination of stained sections revealed that there was no obvious cell atrophy or death caused by DNMT inhibitor infusion (data not shown).

DNMT inhibitor infusion increases anxiety-like behavior

The elevated plus maze was used to assess anxiety behavior in the DNMT inhibitor infused mice. Mice infused with either 5azaC or Zeb spent significantly less time in the open arm ($p < 0.05$; Fig. 2A and 2C) and more time in the closed arm ($p < 0.05$; Fig. 2B and 2D) than their respective vehicle groups, suggestive of an increase in anxiety-related behavior.

Social behavior is unaltered in DNMT inhibitor infused mice

Next, we investigated the impact of DNMT inhibitor treatment on adult social behavior. The experimental mice were placed into an open field arena with a wire mesh cage on one side and allowed to explore for five minutes. Then, the mouse was removed and an adult C57BL/6 mouse was placed behind the wire mesh and the amount of time the experimental mouse interacts with the novel target was assessed. We found that infusion of either 5AzaC or Zeb did not impact the amount of time the experimental animal was near the empty wire mesh (Supplementary Fig. 1). Once an adult C57BL/6 mouse was placed behind the wire mesh we found that, as expected, the vehicle treated animals had a significant increase in time spent in the interaction zone. Similarly, the mice receiving the infusion of either 5AzaC or Zeb had a significant increase in the amount of time spent in the interaction zone when a C57BL/6 mouse was present, suggesting that social behavior was not altered.

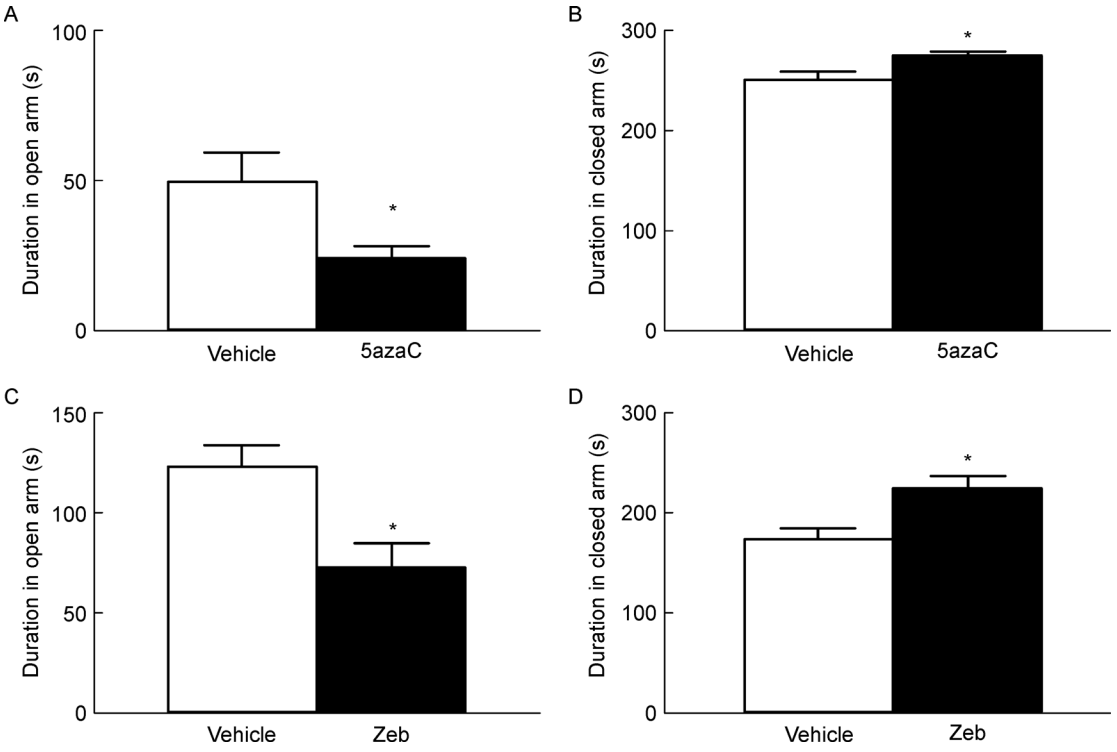

Figure 2 Chronic DNMT inhibitor infusion enhances anxiety-like behavior. (A, C) Mice treated with DNMT inhibitors 5azaC (A) or zebularine (C) spend less time exploring the open arm of the elevated plus maze compared with vehicle-infused animals (*$p < 0.05$). (B, D) Similarly, mice treated with 5azaC (B) or zebularine (D) spend more time in the closed arm of the EPM compared to vehicle-treated mice (*$p < 0.05$).

Locomotor activity is not affected by DNMT inhibitor treatment

In the next set of experiments, we assessed the impact of chronic DNMT inhibitor infusion on locomotor activity. We observed that mice infused with 5azaC were indistinguishable from vehicle-treated mice over a two hour test (Fig. 3A). We also assessed the data in 5 min epochs and there was no significant difference between vehicle and 5aza treatment ($F_{(1-22)} = 1.06$, $p = 0.3137$) (Fig. 3A, inset). Similarly, we observed no difference in activity level between mice infused with Zeb and their vehicle group when examined for total locomotor changes over the a two hour period (Fig. 3B) or when analyzed in 5 min epochs ($F_{(1-22)} = 0.21$, $p = 0.6530$; Fig. 3B, inset).

Fear conditioning is impaired after chronic DNMT inhibitor treatment

To determine if chronic DNMT inhibitor infusion into the dentate gyrus impacts learning and memory, we observed the mice in a fear conditioning paradigm to assesses context dependent learning, which is thought to require partially distinct but overlapping neural circuits (Phillips and LeDoux, 1992; Abel et al., 1997). We examined baseline freezing in the mice and found similar levels in the chronic 5azaC or Zeb treated mice compared to vehicle infused mice (data not shown). However, when re-exposed to the conditioned context 24 h later, mice infused with 5azaC or Zeb showed a significant decrease in freezing compared to their respective vehicle groups ($p < 0.05$; Fig. 4A and 4B) suggestive of a deficit in context dependent fear conditioning induced by DNMT inhibition.

AP5 rescues behavioral deficits and returns Bdnf expression to baseline levels

Our previous *in vitro* data showed that chronic DNMT inhibitor treatment of hippocampal cultures demethylated BDNF gene expression, resulting in an increase in BDNF expression accompanied by alterations in synaptic function that were blocked by the NMDA receptor antagonist, AP5, suggesting a link between DNA methylation and synaptic function. To investigate if this putative link impacts behavior, we examined whether or not AP5 pretreatment would block the behavioral effects of chronic DNMT inhibitor treatment. In these experiments we used a concentration of AP5 that did not give rise to learning and memory deficits by itself (Davis et al., 1992). Separate cohorts of C57BL/6 mice were bilaterally infused in the dentate gyrus with vehicle alone, 5azaC alone, AP5 alone, or AP5 + 5azaC. Mice were infused for 6 days and then tested to see if AP5 could reverse the

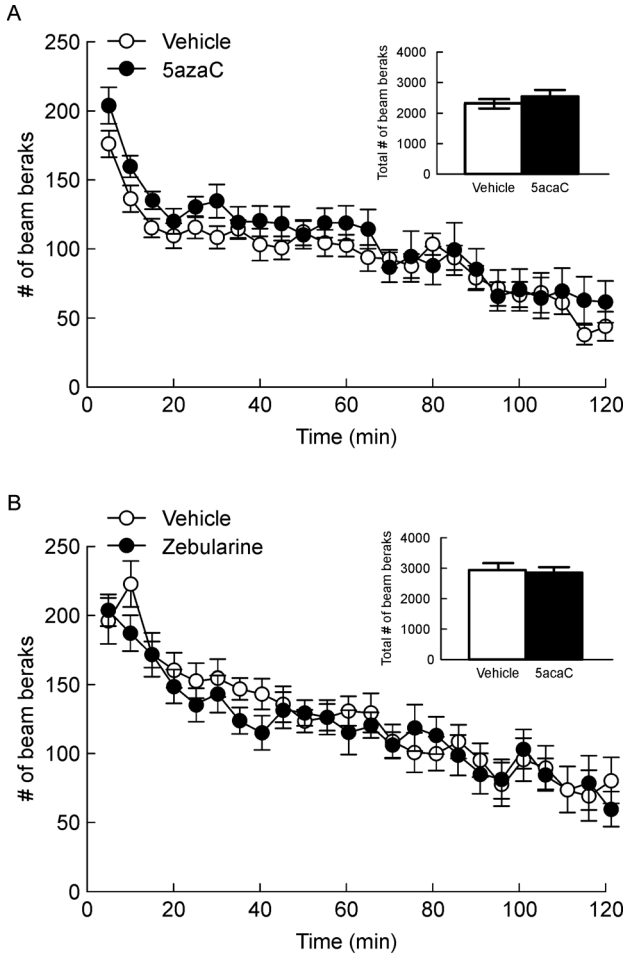

Figure 3 Chronic DNMT inhibitor treatment does not impact locomotor activity. Treatment by 5azaC (A) or zebularine (B) does not alter locomotor behavior .

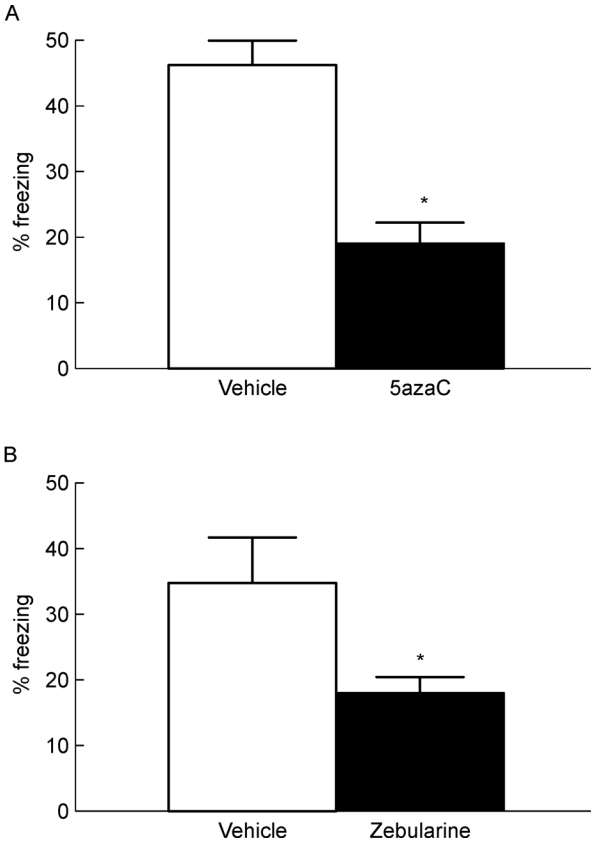

Figure 4 Chronic inhibition of DNMT activity in the dentate gyrus impairs context dependent fear memory. (A,B). Mice receiving (A) 5azaC or (B) zebularine spent significantly less time freezing in the context in which fear training was administered the previous day compared to vehicle-treated mice (*$p < 0.05$).

heightened anxiety and disrupted context dependent fear conditioning phenotypes induced by 5azaC treatment. In initial experiments, we examined these mice for changes in locomotor activity, which could impact behavior, and did not find any alterations in any of the treatment groups (data not shown). We observed behavior in the elevated plus maze and found that in agreement with the previous set of experiments, chronic 5azaC treatment produced significant changes that are suggestive of an increase in anxiety-related behavior ($F_{(3-37)} = 2.852$, $p = 0.05$). We found no change in the time spent in the open arm with AP5 treatment alone. We also found that AP5 treatment reversed these 5azaC induced phenotypes as assessed by Neuman Keul's post-hoc analysis ($p < 0.05$; Fig. 5A). In the fear conditioning paradigm, mice infused with 5azaC showed significant deficits in contextual fear conditioning ($F_{3,38} = 2.739$, $p = 0.05$; Fig. 5B). We observed that 5azaC treatment impaired fear conditioning as assessed by Neuman Keul's post-hoc analysis ($p < 0.05$) while mice infused with both AP5 and 5azaC were indistinguishable from vehicle or AP5 alone treatment.

We examined whether the ability of AP5 to reverse the behavioral deficits due to DNMT inhibition works through a transcriptional-dependent mechanism. We performed quantitative RT-PCR to determine gene expression of candidate target genes BDNF, reelin, and PP1C that have been shown to be actively demethylated in hippocampus (Miller and Sweatt, 2007; Nelson et al., 2008; Ma et al., 2009; Sui et al., 2012). We found a significant effect of drug infusion by one-way ANOVA ($F_{(3,23)} = 3.263$, $p = 0.0429$; Fig. 5C). Post-hoc analysis by Dunnett's test revealed that hippocampal-tissue from 5azaC treated mice showed a significant increase in BDNF mRNA expression compared to vehicle treated samples ($p < 0.05$) while AP5 + 5azaC treatment showed similar expression levels to vehicle-treated mice. Importantly, BDNF expression was not impacted by chronic treatment with AP5 alone. In contrast, there were no significant alterations in the expression of either reelin or PP1C under the conditions examined (Fig. 5C).

Discussion

In this study, we report that chronic DNMT inhibition in the dentate gyrus of hippocampus produces specific behavioral

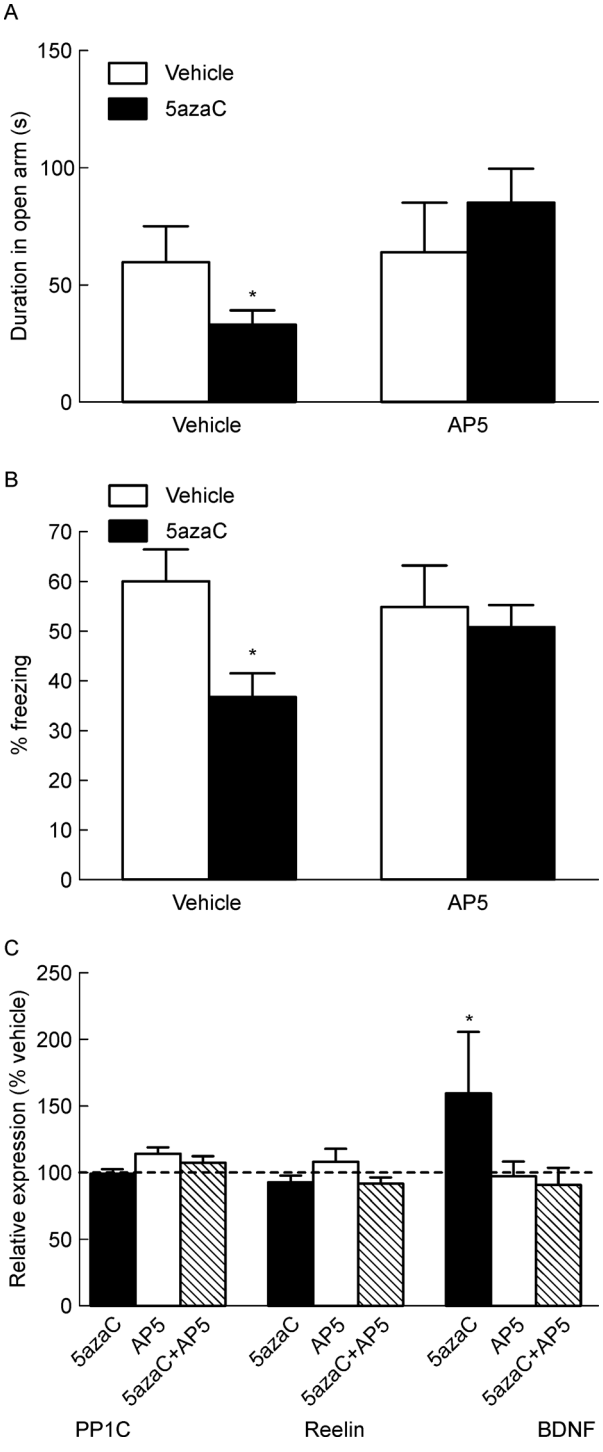

Figure 5 Blockade of excitatory signaling blocks effects of DNMT inhibition. (A) Chronic DNMT inhibition results in increased anxiety-related behavior ($F_{3\text{-}37} = 2.852$, $p = 0.05$, main effect by one-way ANOVA) and this effect is blocked by co-treatment with AP5 (significant post-hoc analysis after Neuman-Keul's correction, 5azaC vs. AP5 + 5azaC groups). (B) As previously shown, DNMT inhibition impairs context-dependent fear memory ($F_{3-37} = 2.759$, $p = 0.05$, main effect by one-way ANOVA, significant post-hoc analysis Neuman-Keul's correction, vehicle vs. 5azaC) while co-treatment of 5azaC + AP5 is indistinguishable from vehicle treatment, though AP5 alone produces no effect on contextual fear memory. (C) Quantitative reverse transcription PCR reveals that DNMT inhibition does not appear to globally enhance transcription, but specifically regulates *Bdnf* levels in the dentate gyrus ($F_{3-23} = 3.263$, $p = 0.04$) (post-hoc analysis by Dunnett's test between vehicle and 5azaC, $^*p < 0.05$). This effect is blocked by antagonism of NMDA receptors by AP5, while AP5 treatment alone does not affect *bdnf* or other transcript levels ($F_{3-23} = 0.3418$, $p = 0.79$ for reelin; $F_{3-23} = 0.6404$, $p = 0.59$ for PP1, one-way ANOVA).

impairments, namely heightened anxiety-related behaviors and deficits in fear conditioning, while resulting in an increase in *Bdnf* expression. Administration of AP5 treatment blocked the anxiety and fear conditioning deficits induced by chronic DNMT inhibition. AP5 treatment also rescued *Bdnf* expression induced by chronic DNMT inhibition suggesting that DNMT inhibition may be working in an activity dependent manner. These results suggest that DNMT inhibition can alter gene expression *in vivo* in a manner that requires coincident NMDA receptor activity. This premise is consistent with recent findings that demonstrated the capacity of neuronal activity to induce DNA breaks (Madabhushi et al., 2015), which in turn would be expected to facilitate 5AzaC and zebularine's ability to impair DNMT-mediated methylation.

Mice administered DNMT inhibitors for 14 days into the dentate gyrus appeared healthy and did not show any differences in weight over of the course of drug infusion. There was no cell loss in the dentate gyrus after inhibitor infusion, in agreement with findings in DNMT 1 and 3a double knockout mice (Feng et al., 2010). Chronic DNMT inhibition did not produce alterations in social interaction behavior or locomotor activity suggesting that chronic DNMT inhibition in the dentate gyrus is not producing global behavioral effects.

Chronic DNMT inhibitor infusion into the dentate gyrus resulted in anxiogenic effects as assessed in the elevated plus maze. Recently, studies have implicated the role of glutamatergic signaling in hippocampus and amygdala in anxiety behavior (Charney and Deutch, 1996; Bergink et al., 2004). A previous study examining the role of dentate gyrus in anxiety behavior revealed that cholecystokinin infusion into this brain region led to activation of PLC and PKC pathways and was associated with increased anxiety-like behavior as assessed by the novelty-suppressed feeding task (Xiao et al., 2012). Intriguingly, enhanced PLC and PKC pathway activation was linked to enhanced NMDA receptor function in the dentate gyrus with no impact on AMPA receptor signaling, suggesting that glutamatergic neuronal activation in the dentate gyrus is regulated during anxiety-related tasks (Xiao et al., 2012).

The finding that DNMT infusion into the DG increased anxiety-related behavior but did not affect social behavior is noteworthy as the potential relationship between anxiety and social deficits is complex with some suggestion that they may share a common neural circuit (Allsop et al., 2014). Indeed, conditional methyl-CpG binding protein 2 (*Mecp2*) mutant mice have heightened anxiety-related behavior and social deficits, recapitulating key aspects of Rett syndrome (Gemelli et al., 2006). However, there are reports in which a dissociation between anxiety and social deficits has also been observed. Selective deletion of *Mecp2* in the basolateral amygdala of mice results in increased anxiety-related behavior with no effect on social interaction suggesting that social interaction deficits are not the result of increased anxiety-related behavior, at least in regards to the selective deletion of *Mecp2* in the BLA (Adachi et al., 2009). Our current finding that chronic DNMT infusion into the DG results in increased anxiety behavior without alterations in social interaction suggests a disassociation between these two behaviors when driven by DNMT inhibition in the DG.

Chronic infusion of the DNMT inhibitors 5azaC and Zeb into the dentate gyrus also led to deficits in context dependent fear conditioning. Previous studies examining the role of DNA methylation in regions of cortex and hippocampus have demonstrated that memory is altered by dysregulation of this process. Acute inhibition of DNMTs in the CA1 region of hippocampus disrupts fear conditioning (Miller and Sweatt, 2007) deletion of DNMT1 and DNMT3a in forebrain neurons impairs fear conditioning as well as Morris water maze behavior (Feng et al., 2010), while more recent data has shown that selective deletion of DNMT3a in forebrain neurons impairs fear conditioning (Morris et al., 2014). Our findings further illustrate the importance of DNA methylation in memory processes and highlight the role of the dentate gyrus specifically in this behavior.

We also observed specific effects of DNMT inhibition on gene expression in the dentate gyrus. Previous studies indicated altered expression of protein phosphatase 1C and reelin in the CA1 was associated with fear conditioning deficits (Miller and Sweatt, 2007). We did not observe this change in PP1C or reelin gene expression with dentate-gyrus infusion of 5azaC or Zeb. Additionally, double knockout mice for both DNMT1 and DNMT3a show no differences in PP1C or reelin expression in the hippocampus (Feng et al., 2010). In fact, knockout of both these DNMTs results in only ~1% decrease in methylation of CpG islands across the entire genome with significant changes appearing at limited regions in specific gene promoters (Feng et al., 2010). Therefore, guided by our previous studies, we also chose to examine *Bdnf* as a candidate gene (Nelson et al., 2008). We saw a significant enhancement of *Bdnf* expression in the dentate gyrus of mice infused chronically with 5azaC. We had previously shown that in cultured dentate gyrus neurons elevated *Bdnf* expression could be reversed by blocking excitatory neurotransmission with AP5 or tetrodatoxin (Nelson et al., 2008). Indeed, we also observed that mice treated with a combination of both AP5 and 5azaC had *Bdnf* expression levels similar to vehicle-infused mice.

Intriguingly, heightened *Bdnf* expression has previously been linked to abnormal behavior. BDNF-Tg mice have forebrain neuronal enhancement of BDNF expression under the CamKII-alpha promoter (Papaleo et al., 2011). These mice show a two-fold enhancement of *Bdnf* expression in the hippocampus, similar to the level of expression we observed in our DNMT inhibitor treated mice. These animals display impairments in spatial memory as assessed by T-maze and a trend toward decreased context dependent fear conditioning. Moreover, the mice also display abnormal anxiety-like behavior as assessed by light-dark testing. These previous data are in agreement with our present findings that

overexpression of *Bdnf* in hippocampus may contribute to altered anxiety and memory.

Our group has previously shown that 5azaC and Zeb specifically impact excitatory neurotransmission by decreasing miniature excitatory postsynaptic currents in cultured neurons (Nelson et al., 2008). In separate work, hippocampal slices treated with 5azaC or Zeb do not achieve long-term potentiation (LTP) although baseline synaptic efficiency is not affected by the treatment (Levenson and Sweatt, 2006). Conditional DNMT1 and 3a double knockouts show a similar synaptic deficit with disruptions in CA1 LTP and LTD (Feng et al., 2010). Recent data characterizing conditional DNMT3a knockout mice have shown that they have deficits in associative learning and in LTP. These changes are not observed in conditional DNMT1 knockout mice, suggesting that *de novo* DNA methylation is necessary for normal memory formation and LTP processes (Morris et al., 2014). Collectively, these studies provide strong support that loss of DNMT function leads to impaired excitatory synaptic plasticity in the hippocampus.

Taken together, we demonstrated that chronic infusion of DNA methyltransferase inhibitors in the dentate gyrus leads to specific behavioral impairments in anxiety-related behavior and fear conditioning associated with enhanced *Bdnf* expression. Our findings that AP5 could block the DNMT inhibitor induced phenotypes and rescue the changes in gene expression suggests that DNMT inhibition enhances gene expression in an activity-dependent manner and that, conversely, the behavior deficits and abnormal gene expression are facilitated by NMDA receptor activity.

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Compliance with ethics guidelines

Megumi Adachi is currently employed by Astellas Research Institute of America. Anita Autry and Lisa Monteggia declare they have no conflict of interest. All institutional and national guidelines for the care and use of Laboratory animals were followed.

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