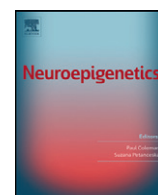




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Original research

DNA methylation regulates neurophysiological spatial representation in memory formation

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ABSTRACT

Epigenetic mechanisms including altered DNA methylation are critical for altered gene transcription subserving synaptic plasticity and the retention of learned behavior. Here, we tested the idea that one role for activity-dependent altered DNA methylation is stabilization of cognition-associated hippocampal place cell firing in response to novel place learning. We observed that a behavioral protocol (spatial exploration of a novel environment) known to induce hippocampal place cell remapping resulted in alterations of hippocampal *Bdnf* DNA methylation. Further studies using neurophysiological in vivo single-unit recordings revealed that pharmacological manipulations of DNA methylation decreased long-term but not short-term place field stability. Together, our data highlight a role for DNA methylation in regulating neurophysiological spatial representation and memory formation.

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Introduction

De novo gene expression within the hippocampus has long been recognized for its necessary role in synaptic plasticity and the long-term retention of learned behavior. Given that memory formation requires experience-driven patterns of gene expression, this has led to the search for molecular mechanisms both adequately sensitive to environmental stimuli and capable of driving and maintaining transcription-dependent cellular changes. As originally proposed by Crick nearly 3 decades ago (Crick, 1984), studies over the past few years have implicated epigenetic mechanisms including DNA methylation as key mediators of memory formation and stabilization. For example, several studies indicate that histone acetylation, an epigenetic change that alters chromatin structure in a manner that promotes gene transcription, plays an important role in mediating hippocampal gene expression associated with contextual fear (Barrett et al., 2011; Miller et al., 2008; Oliveira et al., 2011), object recognition (Barrett et al., 2011; Oliveira et al., 2011), and spatial (Bousiges et al., 2010) memory formation. Methylation of histones

in patterns permissive to gene transcription also supports the formation of contextual fear memory (Gupta et al., 2010; Gupta-Agarwal et al., 2012). Furthermore, consistent with the role of histone modifications in memory, disturbances in hippocampal histone acetylation have been associated with disruptions in hippocampal plasticity and memory capacity in aged rats and in animals with neuronal loss (Fischer et al., 2007; Peleg et al., 2010).

Similarly, studies have shown that active regulation of DNA methylation and demethylation within the hippocampus supports neural plasticity and memory formation. DNA methylation is an epigenetic mechanism typically associated with gene silencing, but studies suggest that it may also be associated with active gene transcription (Chahrour et al., 2008; Uchida et al., 2011; Yasui et al., 2007). Experience-driven changes in gene expression following contextual fear conditioning are associated with methylation of the *PP1* gene (Miller and Sweatt, 2007) and demethylation of the *reelin* (Miller and Sweatt, 2007) and *Bdnf* (Lubin et al., 2008; Mizuno et al., 2012) genes. Changes in DNA methylation and expression of hippocampal *Bdnf* are also associated with object recognition memory (Munoz et al., 2010) and the memory of traumatic experiences (Roth et al., 2011). As further evidence for the role of DNA methylation in memory processes, mice with disruptions in proteins associated with DNA methylation (including MeCP2 and DNMT1) show impairments in long-term potentiation and fear memory formation (Feng et al.,

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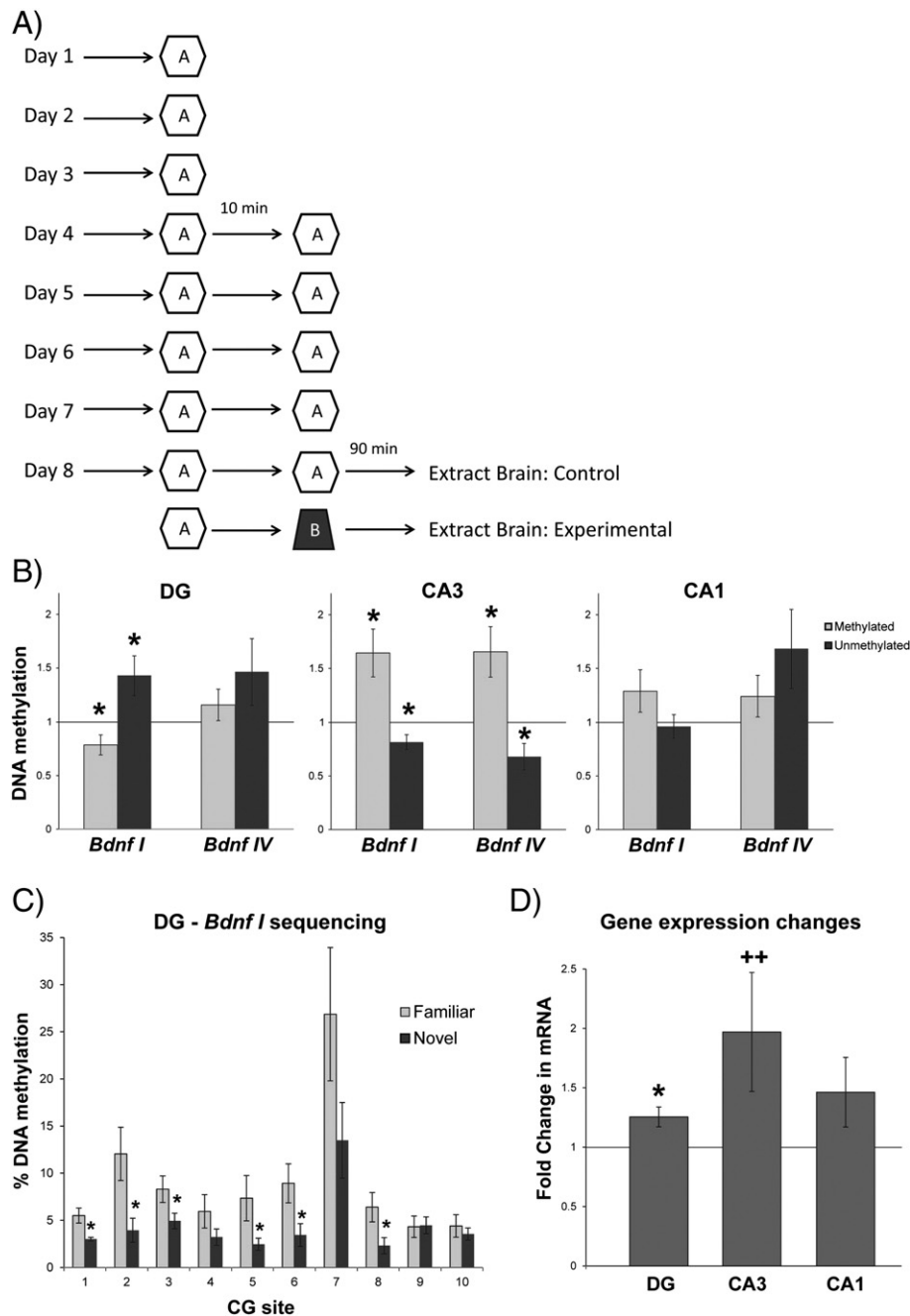


Fig. 1. (A) Graphic depicting the 8-day protocol used in experiment 1. For 8 days, rats performed a foraging task in a familiar environment (environment A). On days 4–8, rats performed the task in two 20-lap sessions separated by 10 minutes. On days 1–7, protocols were identical for the experimental and control group. On day 8, the second 20-lap session for the experimental group was conducted in a novel environment (environment B). Brains were extracted for biochemical analyses 90 minutes after the second foraging session on day 8. (B) Experience-induced alterations of hippocampal *Bdnf* gene DNA methylation. Levels of methylated and unmethylated DNA associated with the *Bdnf* gene in dentate (*Bdnf I* $n = 10$, *Bdnf IV* $n = 11$), CA3 (*Bdnf I* $n = 10$, *Bdnf IV* $n = 11$), and CA1 (*Bdnf I* $n = 11$, *Bdnf IV* $n = 11$) in novel-exposed versus control (familiar-exposed) rats. (C) Methylation analysis of individual CG dinucleotides associated with exon I in the dentate of control and novel-exposed rats ($n = 10$ /group). (D) *Bdnf* mRNA (exon IX) levels in novel-exposed rats relative to familiar-exposed controls (DG $n = 8$, CA3 $n = 9$, CA1 $n = 9$). Error bars represent SEM; * $P < .05$, ** $P = .0885$.

2010; Moretti et al., 2006; Nelson and Monteggia, 2011). Memory dysfunction in aged rats has also been linked to aberrant changes in hippocampal DNA methylation (Penner et al., 2011).

Despite this broad molecular and behavioral background, the means by which chemical modification of DNA might control cognition-associated neuronal circuit firing patterns has been left unaddressed. The general goal of the present study was to investigate the potential contribution of DNA methylation in spatial memory formation and the maintenance of neurophysiological spatial

representations within the hippocampal neural circuit (Moser et al., 2008). A place cell is a hippocampal pyramidal neuron that encodes space by selectively increasing activity (firing rate) in a specific environmental location (place field). A collection of place cells with place fields in different locations may encode large environmental areas, providing the animal with a neurophysiological spatial representation of the environment (O'Keefe and Dostrovsky, 1971), which is commonly termed a *cognitive map* (Tolman, 1948) and believed to play a key role in navigation and spatial learning and memory. To

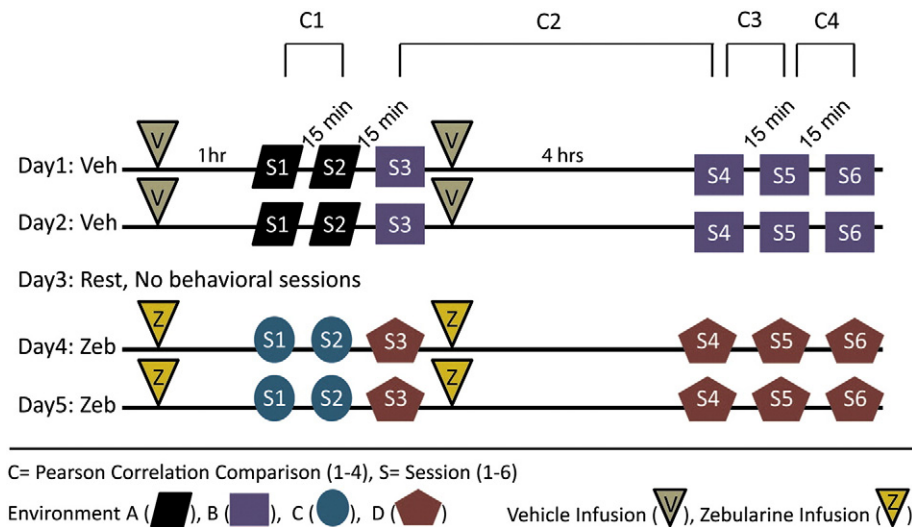


Fig. 2. Graphic depicting the timeline used for the 5-day protocol in experiment 2. Rats performed 6 daily sessions of a foraging task in different environmental setups. A single session consisted of rats completing 13 laps around a circular track. Intracerebroventricular infusions were done 1 hour before session 1 and immediately after session 3. All intersession intervals were approximately 15 minutes (C1, C3, C4) except for the interval (C2) between session 3 and session 4, which was 4 hours.

examine whether DNA methylation is involved in generating or maintaining these maps and related spatial memory processes, we performed 2 experiments. In experiment 1, we examined patterns of DNA methylation in rats that were subjected to a spatial behavioral protocol (Fig. 1A) similar to that used in previous place cell experiments (Roth et al., 2012), where novel environments were shown to induce place cell remapping. Specifically, in our 2012 study, we showed that when a rat transitioned from a familiar to novel environment, some cells maintained a place field in both the familiar and novel environments located at different degrees of rotation around a track (i.e., they rotate), whereas other cells had fields that turned on (appear), turned off (disappear), or split into multiple fields. Together, these data indicate that place fields can exhibit a variety of remapping behaviors. In experiment 2, we pharmacologically manipulated DNA methylation patterns as we performed single-unit in vivo hippocampal place cell recordings, a procedure wherein rats performed behavioral tasks similar to experiment 1, running laps around an elevated track in familiar and novel environments (Figs. 2 and 3).

Results

Experiment 1: assessment of DNA methylation following exposure to a novel environment

If a rat explores a familiar environment, place cell firing patterns remain relatively predictable and stable. However, when a rat explores a novel environment, place cell firing patterns will change (re-map) as new maps and contexts are created or encoded (Bostock et al., 1991; Hill, 1978). If processes generating and maintaining these spatial representations are mediated by DNA methylation, we would then expect DNA methylation patterns to change in response to spatial exposure to a novel environment. In experiment 1, we used a candidate gene approach focused on the *Bdnf* gene to test this prediction. *Bdnf* has been associated with neural plasticity (West et al., 2001), contextual fear conditioning (Hall et al., 2000), and spatial learning and memory (Heldt et al., 2007; Mu et al., 1999). Furthermore, DNA methylation has been implicated in hippocampal *Bdnf* activity-dependent gene regulation (Lubin et al., 2008; Martinowich et al., 2003; Roth et al., 2009, 2011) and consolidation of fear memory (Lubin et al., 2008).

Patterns of DNA methylation in rats that performed the final behavioral session in a novel environment differed significantly from those that performed their final session in a familiar environment (Fig. 1). DNA methylation also varied by subregion and exon of the *Bdnf* gene (Fig. 1B). Data generated using polymerase chain reaction (PCR)-based methylation-specific primers (MSP, see “Materials and methods”) indicated that, for CA3, the novel group had higher levels of methylated DNA associated with *Bdnf* exons I ($t = 2.89, P = .0180$) and IV ($t = 2.78, P = .0195$) relative to familiar controls (Fig. 1B). These results were confirmed with the unmethylated primer set, which indicated lower levels of unmethylated DNA associated with exons I ($t = 2.65, P = .0267$) and IV ($t = 2.61, P = .0263$). No significant changes were found in either methylated or unmethylated DNA levels within CA1. In the dentate (Fig. 1B), the novel group had lower levels of methylated DNA associated with exon I ($t = 2.29, P = .0477$). This is consistent with the observation of higher levels of unmethylated DNA associated with exon I ($t = 2.34, P = .0443$) and our independent replication of this result using direct bisulfite sequencing (BSP) data (Fig. 1C; $F_{1,180} = 22.16, P < .0001$). For all subregions, no significant changes were found in either methylated or unmethylated DNA associated with exon VI (data not shown).

Thus, our MSP and BSP data indicate that DNA methylation and demethylation are evoked by a behavioral paradigm known to induce hippocampal place cell remapping. Because DNA methylation is a known regulator of *Bdnf* gene expression, we examined whether there were corresponding changes in mRNA levels (Fig. 1D). Exon IX-containing transcripts were elevated in the novel group relative to controls in the dentate ($t = 3.05, P = .0186$), and there was also a trend for an increase in *Bdnf* expression in CA3 ($t = 1.94, P = .0885$). These are both regions where we detected changes in DNA methylation. Together, these data indicate that our spatial learning behavioral paradigm does induce changes in *Bdnf* gene expression and that changes in DNA methylation might be one mechanism by which this occurs.

Pharmacological manipulation of DNA methylation alters place field stability

Whereas many place cell studies have focused on defining patterns of plasticity and stability associated with these spatial representations, relatively little is known about the underlying molecular mechanisms. Some of the receptors and transcription factors known

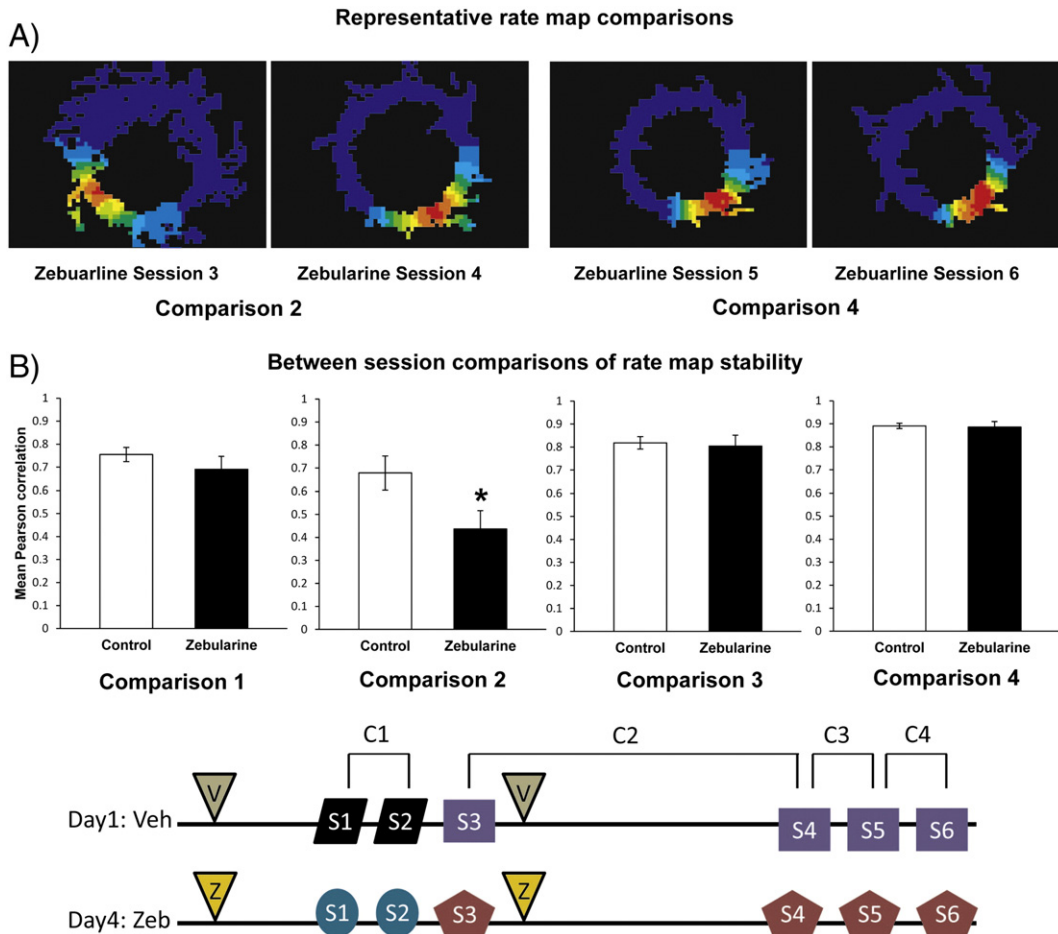


Fig. 3. Pharmacological manipulations of DNA methylation decreased place field stability. (A) Four representative rate maps used for comparisons 2 and 4 in rats treated with zebularine. (B) Mean Pearson correlation coefficients representing place cell rate map stability across behavioral sessions are plotted. Correlations were generated comparing rate maps across sessions 1 and 2, 3 and 4, 4 and 5, and 5 and 6. The light bars represent correlations during the control treatment, and the dark bars represent correlations during the zebularine treatment. Correlations between the control and zebularine treatment significantly differ ($*P < .05$) in the session 3 to session 4 (3–4) comparison. Error bars represent SEM.

to be involved in long-term potentiation and memory consolidation have been linked to place cell encoding and stability, which include hyperpolarization-activated cyclic nucleotide-gated channels (Hussaini et al., 2011; Nolan et al., 2004), NMDA receptors (Ekstrom et al., 2001; Kentros et al., 1998; Nakazawa et al., 2003), and *zif268* (Renaudineau et al., 2009). Together, these studies highlight the importance of cell-signaling cascades and gene activity to place cell function; but epigenetic factors underlying transcriptional regulation and associated gene expression patterns in spatial cognition remain unclear. If place cell properties and learning and memory processes associated with spatial representations are mediated through DNA methylation mechanisms, we would predict that manipulations of DNA methylation patterns would alter place cell firing patterns.

To assess this, in experiment 2, we pharmacologically manipulated DNA methylation patterns as we performed single-unit in vivo hippocampal place cell recordings (see Fig. 2 for the basic experimental design). For this experiment, we used a procedure wherein rats performed behavioral tasks similar to experiment 1, running laps around an elevated track in familiar and novel environments. We then measured the stability of hippocampal place cell firing rate patterns (rate maps) by making 4 pairwise comparisons across 6 different time points of an extended spatial learning and memory episode. For each of the 4 rate map comparisons (sessions 1 to 2, 3 to 4, 4 to 5,

and 5 to 6; Figs. 2 and 3) for experiment 2, place field stability over time was examined using Pearson correlations for both the drug and control treatments. Each correlation comparison was calculated from an average total of 55.5 ± 5.48 (SE) place cells from the 5 rats. Although we recorded from both CA1 and CA3 cells, most recording tetrodes were placed in CA1, resulting in 78% of correlation values. Correlations from both CA1 and CA3 cells were statistically similar in session comparisons. In all analyses of variance (ANOVAs), the main effect and all interaction effects associated with the factor hippocampal subregion (CA1, CA3) were highly nonsignificant. Thus, CA1 and CA3 cells were collapsed into 1 group; and subregion was removed from the statistical model. Results (Fig. 3B) are reported for a 2-way ANOVA examining the effects of drug treatment (zebularine, vehicle) and session comparison (1–2, 3–4, 4–5, 5–6) on rate map stability (Pearson correlation values). We observed significant main effects for both drug treatment ($F_{1,436} = 7.39, P = .0068$) and session comparison ($F_{3,436} = 19.18, P < .0001$), as well as a marginally significant drug treatment by session comparison interaction effect ($F_{3,436} = 2.57, P = .0538$). In comparisons across all 4 sessions, mean rate map correlations for the zebularine treatment were less than controls (Fig. 3B); but in within-session comparison, the differences between drug treatments were not statistically significant except in the 3–4 (long-term novel to familiar) session comparison where correlations in the zebularine treatment were significantly

lower ($F_{1,64} = 4.81, P = .032$). Post hoc Fisher protected least significant difference pairwise comparisons revealed that all session comparisons were significantly different (all P s < .05) from each other. As would be expected, rate map correlations were generally lower in both zebularine and control treatments in the novel to familiar session comparisons (1–2, 3–4) and then generally increased as familiarity with the environment increased with multiple exposures (sessions 4–5, 5–6). As this familiarity with the environment increased from session 3 to session 6, the mean differences between the drug and control groups decreased. Overall, these data indicate a significant destabilization of hippocampal place cell firing patterns, in a within-subject design \pm drug, upon administration of a DNMT inhibitor, an observation consistent with the hypothesis that DNA methylation contributes to regulating the stability of hippocampal place cell firing patterns.

Discussion

In experiment 1, we predicted that in behavioral paradigms known to induce hippocampal place cell remapping, we would also observe alterations to DNA methylation patterns as novel environments are encoded or learned. Indeed, the rats that performed the final behavioral session in a novel environment showed changes in patterns of hippocampal DNA methylation compared with controls. Specifically, rats with the novel spatial experience exhibited decreased *Bdnf* DNA methylation in the dentate gyrus and increased *Bdnf* DNA methylation in CA3. A rat's spatial experience likely involves a complex interaction of contextual learning elements, exercise, and stress, factors on their own capable of inducing epigenetic modifications and altering gene expression (Bilang-Bleuel et al., 2005; Chandramohan et al., 2007; Lubin et al., 2008; Penner et al., 2011; Roth et al., 2011). Rats were overtrained for an extended period of time in the same context to try to minimize the influence of contextual learning and other factors associated with the behavioral task. To control for exercise, the number of laps during the behavioral paradigm were held constant between the 2 groups of animals. Although our animals did not appear stressed, it is possible that our changes in DNA methylation patterns also reflect a response to a stressor (novel environment) rather than a spatial learning and memory mechanism per se, or perhaps both because spatial learning and stress are likely not mutually exclusive processes. Nonetheless, these results provide the first demonstration that a novel spatial experience can alter DNA methylation patterns in the hippocampus in a subregion and exon-specific manner for a given gene.

In experiment 2, we directly recorded the neurobiological hippocampal circuit spatial representation while pharmacologically altering DNA methylation. Before experimentation, we had very little detailed knowledge about what, where, and when spatial learning and memory machinery and associated gene expression might be affected by DNA methylation. We did know from our previous in vivo work with zebularine that it is capable of altering hippocampal DNA methylation and gene expression patterns 40 minutes post-infusion (Lubin et al., 2008). To increase our chances of detecting changes in spatial representation in response to manipulation of methylation, experiments were designed to examine many different contexts and time points. For session comparison 1–2, session 1 represents the rat's first exposure to a novel environment. After a brief 15-minute rest, session 2 represents the rat's second exposure to that same environment. Here, we tested if an intracerebroventricular (ICV) infusion of a demethylating agent 1 hour before session 1 alters the encoding or acquisition of a spatial representation (map) and/or the reexpression of this map in session 2. Under these conditions, we did not see any significant effects of the drug on place field stability

compared with control trials (Fig. 3). These data indicate that the zebularine infusion does not nonspecifically disrupt the ability of the hippocampal circuit to form a place field firing pattern. For session comparison 3–4, session 3 also represents the rat's first exposure to another novel environment and session 4 is the first reexposure to this environment. This is similar to the 1–2 comparison except that there is a 4-hour delay between sessions. Under these long-term delay conditions, we did see significant decreases in place field stability in DNMT inhibitor-treated rats compared with control trials. The session 4–5 and 5–6 comparisons were reexposures to the 3–4 environment, but the delay between sessions was reduced to 15 minutes. Under these conditions, place field stability returned to levels similar to control trials. In summary, we only detected a drug effect on place field stability when a long delay period was used between sessions. These data suggest that DNA methylation controls place field stabilization over time but is not necessary for initial map formation.

These short-term versus long-term results coincide well with previous reports. Injections of anisomycin, a protein synthesis blocker, revealed that protein synthesis was necessary for long-term (6 hours between sessions) place field stability but not necessary for short-term stability (1 hour between sessions) (Agnihotri et al., 2004). Additionally, injections of CPP, a competitive NMDA receptor antagonist, did not significantly disrupt short term place field stability (1.5 hours) while abolishing long-term stability (16–24 hours) (Kentros et al., 1998). Mice lacking the transcription factor *zif268* exhibited normal short-term (1 hour) place field stability, but long-term (24 hours) stability was disrupted (Renaudineau et al., 2009). Under our current protocol, the ability to form stable fields in the short-term comparisons (1–2, 4–5, 5–6) before and after the long-term comparison (3–4) suggests that the drug effect in session 3–4 comparison likely represents a disruption of a memory consolidation mechanism rather than acquisition. Similarly, our study suggests that long-term place field stability (4 hours) may require molecular memory processes that are influenced by epigenetic mechanisms that include DNA methylation.

It is important to note that in our within-subjects design, the drug treatment always came after the control treatment because we were concerned that effects from the drug may otherwise carry over into the control treatment. Thus, we cannot experimentally rule out sequence effects. However, the significant loss of place field stability in the 3–4 comparison in the drug treatment is likely not a sequence effect. Nothing in the place field literature would predict this instability from a sequence effect. Actually, if any sequence effects were present, we would expect the reverse to happen, as place field stability is more likely to increase as the animal becomes more experienced with the task. Finally, for future studies, another important factor to consider relative to timing and consolidation processes is that the second daily ICV infusion occurred immediately after the third session. It is unclear if this infusion was necessary to disrupt consolidation processes between sessions 3 and 4 or if the first daily infusion was sufficient to produce this effect.

In summary, our results support the hypothesis that DNA methylation plays a role in regulating neurophysiological correlates of space and spatial memory formation (Feng et al., 2010; Lubin et al., 2008; Miller and Sweatt, 2007). To our knowledge, this is the first study to demonstrate that a novel spatial experience alters DNA methylation patterns that vary by subregion and exon of the *Bdnf* gene in the hippocampus. Likewise, we provide novel evidence regarding a potential role of DNA methylation in place field stability and spatial memory formation. Although many questions remain and additional research is needed to provide detailed mechanistic explanations, this study provides valuable insight into the potential molecular mechanisms influencing long-term stability of neurobiological spatial representations and memory. Additionally, given that DNA methylation controls memory capacity in aging (Oliveira et al., 2012), this work provides support

for the speculative notion that the decline in spatial memory associated with aging and neurological disease could reflect dysregulation of hippocampal circuit function due to alterations in DNA methylation.

Materials and methods

Subjects

All 3 experiments used adult male Long-Evans rats (400–500 g). Rats were housed individually in plastic cages with a 12-hour day and night cycle, with food and water provided ad libitum. In experiments 1 and 2, approximately 2 weeks before beginning behavioral experiments, food was restricted to maintain rats at 85%–90% of their ad libitum weights (but ad libitum access to water remained). All procedures were approved by University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Apparatus and training

In experiments 1 and 2, rats performed a foraging task. To perform this task, rats were trained to run laps clockwise around an elevated circular track as they foraged for chocolate sprinkles randomly placed on the track by an experimenter. Although tracks were painted different colors and had different textures in different environmental setups, all tracks were the same size (76 cm outer diameter, 55 cm inner diameter) and mounted 13 cm above a platform that was 75 cm above the floor.

Histology

To confirm tetrode and/or cannula locations, transcatheter perfusions (10% formalin) were performed after experiments 2 and 3. Brains were removed and placed in 30% sucrose formalin solution. Brains were sectioned at 40 μm on a microtome, mounted, and stained with cresyl violet. To aid in confirming tetrode locations after experiment 2, approximately 24 hours before perfusions, electrolytic lesions were generated (10 μA for 15 seconds) on a subset of tetrodes.

Drug infusions

Experiment 2 included ICV drug infusions of zebularine to manipulate DNA methylation. Zebularine is a demethylating agent known to reverse DNA methylation (Cheng et al., 2003; Marquez et al., 2005) and has been used to alter experience-dependent DNA methylation changes in other paradigms (Lubin et al., 2008; Roth et al., 2009). For all infusions, a guide cannula was placed in the left lateral ventricle; and 3 μL (infusion rate of 1 $\mu\text{L}/\text{min}$) of either vehicle (control treatment: 10% dimethyl sulfoxide [DMSO]) or zebularine (drug treatment: 600 ng/ μL in 10% DMSO) was delivered via a microinfusion pump.

Experiment 1: assessment of DNA methylation following exposure to a novel environment

Behavioral training

Speakers were placed under the platform to provide background noise. Two behavioral rooms that differed in environmental cues (differences in visual cues, floor and ceiling patterns, platform texture, and track type) were used. One room served as the training environment (thus labeled *familiar*), whereas the other room was used as the novel environment. Rooms were randomly assigned as the familiar or novel environment before training. During the 3-day training period, 22 male Long-Evans rats were trained (30 min/d) on the foraging task on the circular track in the familiar environment. The rats were also familiarized with a staging location where they were permitted to rest in a small circular dish between sessions.

Behavioral testing

After training, 11 rats were assigned to the control group and 11 were assigned to the experimental group. On any given experimental day, the same general experimental procedure was followed (Fig. 1A). Each rat in the control group was first transported in an enclosed opaque plastic box (disorientation chamber) from the home cage to the staging location or resting dish and permitted to habituate in the dish for 5 minutes. The rat was then placed at a random start location on the track in the familiar training environment. After completing 20 laps of the foraging task, the rat was returned to the disorientation box and walked around the laboratory. Tracks were cleaned with 70% ethanol between all behavioral sessions. After a 10-minute rest period, the rat was again placed on the track for a second 20-lap session in the familiar environment. These 2 daily behavioral sessions in the familiar environment were repeated for 5 days. For the rats in the novel group, the protocol was identical except that the second behavioral session on the fifth day was conducted in the novel behavioral room. Ninety minutes after the second behavioral session on the fifth day, each rat was retrieved from its home cage and its brain was removed for biochemical analyses.

Biochemical analyses

Ninety minutes following completion of the spatial protocol, brains were removed, sectioned (1 mm), and brushed onto glass slides; and the tissue was stored at -80°C . DNA was extracted from the hippocampal subregions (dentate gyrus, CA3, CA1) using an AllPrep DNA/RNA kit (Qiagen). Following bisulfite treatment (Qiagen EpiTect kit), methylation status was assessed via methyl-specific real-time PCR (MSP) in a Bio-Rad iQ5 system. The PCR primer sequences designed to detect methylated versus unmethylated DNA within target *Bdnf* exons (I, IV, VI) were used as previously described (Lubin et al., 2008; Roth et al., 2009). Product specificity was confirmed via a melting curve analysis performed in increasing 1°C increments (real-time PCR) or gel electrophoresis. Samples were normalized to tubulin, and the comparative Ct method and 2-tailed 1-sample *t* tests were used to calculate differences in methylation in animals from the novel versus familiar group.

For a subset of samples, methylation status of exon I was also independently assessed via BSP using a similar protocol as previously described (Lubin et al., 2008; Parrish et al., 2012; Roth et al., 2009, 2011). Bisulfite-treated samples were amplified by the following primer sequences: 5'-TTT ATT TTT TGG AGT TTG TGG TAT G-3' (forward) and 5'-ACT TCT CAA ATA AAA ATT AAC AAC CTC TAT-3' (reverse). The PCR products were purified using a gel extraction kit (Qiagen) and sequenced using the reverse primer. The percentage methylation of each CG site within the amplified region was determined by the ratio between peak values of G and A ($G/[G + A]$) on the electropherograms (determined using Chromas software). Universally unmethylated and methylated standards (EpigenDx) were run in parallel with samples, which indicated that the ratio of cytosine methylation increased proportionately with expected methylation rates ($r^2 = 0.9$, $P < .001$). Differences in BSP data were analyzed by analysis of variance tests with Bonferroni post hoc tests when appropriate.

To determine whether there were changes in *Bdnf* gene expression consistent with methylation changes, RNA was extracted from the same tissue using an AllPrep DNA/RNA kit (Qiagen). Reverse transcription was performed using a cDNA synthesis kit (Qiagen). cDNA was amplified by real-time PCR (Bio-Rad iQ5 system) using Taqman probes targeted at exon IX (Applied Biosystems), with tubulin serving as a reference gene. The comparative Ct method was used to calculate differences in gene expression between samples, and group differences in mRNA levels were analyzed by 2-tailed 1-sample *t* tests.

Experiment 2: hippocampal single unit recordings

Surgical procedures

Five male rats were anesthetized by isoflurane inhalation, and a microdrive array was implanted above the right dorsal hippocampus (3.9 mm posterior to bregma, 3.6 mm lateral to midline). The microdrive array was made up of 20 recording probes or tetrodes. A stainless steel guide cannula (23 gauge) was placed in the left lateral ventricle (1.4 mm posterior to bregma, 2.1 mm lateral from midline, and 3.0 mm ventral to brain surface) for drug infusions. The guide cannula and microdrive were secured with stainless steel screws and dental cement. Rats were allowed 1 week to recover before further behavioral training.

Single-unit neuronal recordings

Tetrodes were constructed with 4 fine (0.0005 inch) insulated nichrome electrode wires twisted together. Each electrode was goldplated to obtain an impedance between 200 and 300 k Ω measured at 1 kHz. Neuronal signals were passed through a headstage (HS 36, Neuralynx) to a data acquisition system (Digital Lynx, Neuralynx) and filtered between 600 Hz and 6 kHz. Light-emitting diodes mounted on the headstage were used to track the animal's location at 60 Hz.

Behavioral training

Similar to experiment 1, rats were trained on the foraging task. Generally, 9–16 training sessions were required before the experiment to ensure that rats could meet the behavioral criteria (i.e., attentively foraging for sprinkles with limited distractions, completing at least 15 laps within 10 minutes). Rats were also familiarized with another room (quiet room) where rats were permitted to rest in a small circular dish. During the postsurgical training period (10–18 days), electrodes were gradually advanced to pyramidal cell layers in CA1 and CA3.

Behavioral apparatus and environment

The behavioral recording environment was cylindrical (2.3 m diameter) with the outer perimeter defined by curtains extending from floor to ceiling. The platform with a circular track was located in the center of the room. Light was provided by a single 25-W bulb mounted in the ceiling in the center of the room. A commutator with recording tethers and a video camera were ceiling mounted slightly offset from center (≤ 12 cm) surrounding the central light. Speakers were mounted behind the ring of curtains in the 4 corners of the room to provide background noise. By altering floor, ceiling, platform, and wall coverings, as well as exchanging circular tracks and directional audio cues, 4 distinct environments were created for behavioral testing. Environmental pairings were randomly determined for each rat. The first environment that the rats experienced was always labeled as environment A (Env.A), and the second environment was labeled as environment B (Env.B).

Behavioral testing

The within-subject experimental design contained a control treatment (infusion of DMSO) and a drug treatment (infusion of Zebularine). The drug and control treatments consisted of 2 days of single-unit recordings during multiple sessions of the foraging task. Each foraging task session consisted of the rat completing 13 laps around the track. Each of the 5 rats sequentially received 2 days of the control treatment followed by a day of rest (with no behavioral tasks) followed by 2 days of drug treatment.

On any given experimental day, the same general experimental procedure was followed (Fig. 2). Each day was split into 2 sets (morning and afternoon) of 3 behavioral (13 laps) recording sessions (i.e., a total of 6 behavioral recording sessions per day). The 2 sets were separated by 4 hours, whereas the 3 within-set behavioral recording

sessions were separated by approximately 15 minutes. The first 2 sessions were in the same environment (e.g., Env.A). The third through sixth sessions were in another environment (e.g., Env.B). Rats were infused 1 hour before the first session and immediately after the third session. After the rats received the first infusion, they were permitted to rest quietly in a dish in a quiet room as we performed single-unit recordings to collect baseline sleep data for approximately 20 minutes. These data were used to assess recording stability. One hour after infusion, the rat was placed in a covered box and briefly walked around the laboratory for disorientation before entering the behavior room. Once in the behavior room (e.g., Env.A), the rat was removed from the disorientation box and placed on a pedestal in the center of the track and left undisturbed for 2 minutes. We then connected the rat's microdrive to the Neuralynx headstage and tethers that transmit signals to the acquisition system. The rat was then placed at a random start location on the track to begin session 1 as we recorded neuronal signals and tracked the rat's location. After completing 13 laps, the rat was returned to the disorientation box and transported to the dish in the quiet room. The rat rested in the dish for 15 minutes and was then placed back in the disorientation box.

This process was repeated for sessions 2 and 3. However, between sessions 2 and 3, the behavioral room was converted to the other environmental setup (e.g., from Env.A to Env.B.). After all 3 behavioral sessions were completed in the first (morning) set, another (5 minutes) baseline sleep data session was recorded and the rat was infused for the second time. In the afternoon set (4 hours later), this protocol was repeated. However, all 3 behavioral sessions were in the same environment as the third session (e.g., Env.B), no infusions were administered, and the final baseline sleep data session was 20 min. Drug and control treatments used the same protocols except that each treatment had a unique set of behavioral environments (e.g., treatment 1 control: Env.A/Env.B, treatment 2 drug: Env.C/Env.D).

Data analyses

Custom software was used to isolate single units by examining relative signal amplitudes and other waveform parameters on each tetrode wire. Spatial information scores were calculated (Skaggs et al., 1993). A place cell was defined as any cell with a statistically significant ($P \leq .05$) information score of ≥ 0.7 with ≥ 100 spikes. Rate maps were generated for each cell in each session by dividing the number of spikes in a pixel by the amount of time spent in each pixel. Rate maps were smoothed as 640–480 pixel arrays were collapsed into 64–48 bins (bin size, 2.2 cm \times 2.2 cm). Spatial correlations (Pearson) were calculated between rate map distributions in sessions 1 and 2, 3 and 4, 4 and 5, as well as 5 and 6. Correlation coefficients were only generated if a cell met the criteria above defining a place cell in at least one of the sessions being compared and generated at least 50 spikes in the other session. These Pearson correlations were analyzed by ANOVA with session comparison (session1–2, 3–4, 4–5, 5–6), drug (vehicle, zebularine), and subregion (CA1, CA3) as factors. To avoid potential confounding interactions across days, analyses were limited to comparisons of day 1 of the drug and control treatment.

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Authors EDR, TLR, and JDS designed the study and wrote the paper. For experiment 1, EDR performed the behavioral training and TLR performed the biochemical analyses. For experiment 2, EDR, KMM, SS, and DEE performed the surgeries, behavioral training, and neuronal recordings. All authors took part in the analysis of data and interpretation of the results.

Disclosure statement

The authors report no biomedical financial interests or potential conflicts of interest.

References

- Agnihotri, N.T., Hawkins, R.D., Kandel, E.R., Kentros, C., 2004. The long-term stability of new hippocampal place fields requires new protein synthesis. *Proc Natl Acad Sci U S A* 101, 3656–3661.
- Barrett, R.M., Malvaez, M., Kramar, E., Matheos, D.P., Arrizon, A., Cabrera, S.M., Lynch, G., Greene, R.W., Wood, M.A., 2011. Hippocampal focal knockout of CBP affects specific histone modifications, long-term potentiation, and long-term memory. *Neuropsychopharmacology* 36, 1545–1556.
- Bilang-Bleuel, A., Ulbricht, S., Chandramohan, Y., De Carli, S., Droste, S.K., Reul, J.M., 2005. Psychological stress increases histone H3 phosphorylation in adult dentate gyrus granule neurons: involvement in a glucocorticoid receptor-dependent behavioural response. *Eur J Neurosci* 22, 1691–1700.
- Bostock, E., Muller, R.U., Kubie, J.L., 1991. Experience-dependent modifications of hippocampal place cell firing. *Hippocampus* 1, 193–205.
- Bousiges, O., de Vasconcelos, A.P., Neidl, R., Cosquer, B., Herbeaux, K., Panteleeva, I., Loeffler, J.P., Cassel, J.C., Boutillier, A.L., 2010. Spatial memory consolidation is associated with induction of several lysine-acetyltransferase (histone acetyltransferase) expression levels and H2B/H4 acetylation-dependent transcriptional events in the rat hippocampus. *Neuropsychopharmacology* 35, 2521–2537.
- Chahrouh, M., Jung, S.Y., Shaw, C., Zhou, X., Wong, S.T., Qin, J., Zoghbi, H.Y., 2008. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* 320, 1224–1229.
- Chandramohan, Y., Droste, S.K., Reul, J.M., 2007. Novelty stress induces phosphorylation of histone H3 in rat dentate gyrus granule neurons through coincident signalling via the N-methyl-D-aspartate receptor and the glucocorticoid receptor: relevance for c-fos induction. *J Neurochem* 101, 815–828.
- Cheng, J.C., Matsen, C.B., Gonzales, F.A., Ye, W., Greer, S., Marquez, V.E., Jones, P.A., Selker, E.U., 2003. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J Natl Cancer Inst* 95, 399–409.
- Crick, F., 1984. Memory and molecular turnover. *Nature* 312, 101.
- Ekstrom, A.D., Meltzer, J., McNaughton, B.L., Barnes, C.A., 2001. NMDA receptor antagonism blocks experience-dependent expansion of hippocampal "place fields". *Neuron* 31, 631–638.
- Feng, J., Zhou, Y., Campbell, S.L., Le, T., Li, E., Sweatt, J.D., Silva, A.J., Fan, G., 2010. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci* 13, 423–430.
- Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M., Tsai, L.H., 2007. Recovery of learning and memory is associated with chromatin remodelling. *Nature* 447, 178–182.
- Gupta, S., Kim, S.Y., Artis, S., Molfese, D.L., Schumacher, A., Sweatt, J.D., Paylor, R.E., Lubin, F.D., 2010. Histone methylation regulates memory formation. *J Neurosci* 30, 3589–3599.
- Gupta-Agarwal, S., Franklin, A.V., Deramus, T., Wheelock, M., Davis, R.L., McMahon, L.L., Lubin, F.D., 2012. G9a/GLP histone lysine dimethyltransferase complex activity in the hippocampus and the entorhinal cortex is required for gene activation and silencing during memory consolidation. *J Neurosci* 32, 5440–5453.
- Hall, J., Thomas, K.L., Everitt, B.J., 2000. Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nat Neurosci* 3, 533–535.
- Heldt, S.A., Stanek, L., Chhatwal, J.P., Ressler, K.J., 2007. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol Psychiatry* 12, 656–670.
- Hill, A.J., 1978. First occurrence of hippocampal spatial firing in a new environment. *Exp Neurol* 62, 282–297.
- Hussaini, S.A., Kempadoo, K.A., Thuault, S.J., Siegelbaum, S.A., Kandel, E.R., 2011. Increased size and stability of CA1 and CA3 place fields in HCN1 knockout mice. *Neuron* 72, 643–653.
- Kentros, C., Hargreaves, E., Hawkins, R.D., Kandel, E.R., Shapiro, M., Muller, R.V., 1998. Abolition of long-term stability of new hippocampal place cell maps by NMDA receptor blockade. *Science* 280, 2121–2126.
- Lubin, F.D., Roth, T.L., Sweatt, J.D., 2008. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *J Neurosci* 28, 10576–10586.
- Marquez, V.E., Kelley, J.A., Agbaria, R., Ben-Kasus, T., Cheng, J.C., Yoo, C.B., Jones, P.A., 2005. Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. *Ann N Y Acad Sci* 1058, 246–254.
- Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., Fan, G., Sun, Y.E., 2003. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* 302, 890–893.
- Miller, C.A., Sweatt, J.D., 2007. Covalent modification of DNA regulates memory formation. *Neuron* 53, 857–869.
- Miller, C.A., Campbell, S.L., Sweatt, J.D., 2008. DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. *Neurobiol Learn Mem* 89, 599–603.
- Mizuno, K., Dempster, E., Mill, J., Giese, K.P., 2012. Long-lasting regulation of hippocampal Bdnf gene transcription after contextual fear conditioning. *Genes Brain Behav* 11, 651–659.
- Moretti, P., Levenson, J.M., Battaglia, F., Atkinson, R., Teague, R., Antalfy, B., Armstrong, D., Arancio, O., Sweatt, J.D., Zoghbi, H.Y., 2006. Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. *J Neurosci* 26, 319–327.
- Moser, E.I., Kropff, E., Moser, M.B., 2008. Place cells, grid cells, and the brain's spatial representation system. *Annu Rev Neurosci* 31, 69–89.
- Mu, J.S., Li, W.P., Yao, Z.B., Zhou, X.F., 1999. Deprivation of endogenous brain-derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. *Brain Res* 835, 259–265.
- Munoz, P.C., Aspe, M.A., Contreras, L.S., Palacios, A.G., 2010. Correlations of recognition memory performance with expression and methylation of brain-derived neurotrophic factor in rats. *Biol Res* 43, 251–258.
- Nakazawa, K., Sun, L.D., Quirk, M.C., Rondi-Reig, L., Wilson, M.A., Tonegawa, S., 2003. Hippocampal CA3 NMDA receptors are crucial for memory acquisition of one-time experience. *Neuron* 38, 305–315.
- Nelson, E.D., Monteggia, L.M., 2011. Epigenetics in the mature mammalian brain: effects on behavior and synaptic transmission. *Neurobiol Learn Mem* 96, 53–60.
- Nolan, M.F., Malleret, G., Dudman, J.T., Buhl, D.L., Santoro, B., Gibbs, E., Vronskaya, S., Buzsaki, G., Siegelbaum, S.A., Kandel, E.R., et al., 2004. A behavioral role for dendritic integration: HCN1 channels constrain spatial memory and plasticity at inputs to distal dendrites of CA1 pyramidal neurons. *Cell* 119, 719–732.
- O'Keefe, J., Dostrovsky, J., 1971. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res* 34, 171–175.
- Oliveira, A.M., Estevez, M.A., Hawk, J.D., Grimes, S., Brindle, P.K., Abel, T., 2011. Subregion-specific p300 conditional knock-out mice exhibit long-term memory impairments. *Learn Mem* 18, 161–169.
- Oliveira, A.M.M., Hemstedt, T.J., Bading, H., 2012. Rescue of aging-associated decline in Dnmt3a2 expression restores cognitive abilities. *Nat Neurosci* 15, 1111–1113.
- Parrish, R.R., Day, J.J., Lubin, F.D., 2012. Direct bisulfite sequencing for examination of DNA methylation with gene and nucleotide resolution from brain tissues. *Curr Protoc Neurosci* (Unit 7 24) (Chapter 7).
- Peleg, S., Sananbenesi, F., Zovoilis, A., Burkhart, S., Bahari-Javan, S., Agis-Balboa, R.C., Cota, P., Wittmann, J.L., Gogol-Doering, A., Opitz, L., et al., 2010. Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* 328, 753–756.
- Penner, M.R., Roth, T.L., Chawla, M.K., Hoang, L.T., Roth, E.D., Lubin, F.D., Sweatt, J.D., Worley, P.F., Barnes, C.A., 2011. Age-related changes in Arc transcription and DNA methylation within the hippocampus. *Neurobiol Aging* 32, 2198–2210.
- Renaudineau, S., Poucet, B., Laroche, S., Davis, S., Save, E., 2009. Impaired long-term stability of CA1 place cell representation in mice lacking the transcription factor zif268/egr1. *Proc Natl Acad Sci U S A* 106, 11771–11775.
- Roth, T.L., Lubin, F.D., Funk, A.J., Sweatt, J.D., 2009. Lasting epigenetic influence of early-life adversity on the BDNF gene. *Biol Psychiatry* 65, 760–769.
- Roth, T.L., Zoladz, P.R., Sweatt, J.D., Diamond, D.M., 2011. Epigenetic modification of hippocampal Bdnf DNA in adult rats in an animal model of post-traumatic stress disorder. *J Psychiatr Res* 45, 919–926.
- Roth, E.D., Yu, X., Rao, G., Knierim, J.J., 2012. Functional differences in the backward shifts of CA1 and CA3 place fields in novel and familiar environments. *PLoS One* 7, e36035.
- Skaggs, W.E., McNaughton, B.L., Gothard, K.M., Markus, E.J., 1993. An information-theoretic approach to deciphering the hippocampal code. In: Hanson, S.J., Cowan, J.D., Giles, C.L. (Eds.), *Advances in Neural Information Processing Systems*. Morgan Kaufman, San Mateo, CA, pp. 1030–1037.
- Tolman, E.C., 1948. Cognitive maps in rats and men. *Psychol Rev* 55, 189–208.
- Uchida, S., Hara, K., Kobayashi, A., Otsuki, K., Yamagata, H., Hobaru, T., Suzuki, T., Miyata, N., Watanabe, Y., 2011. Epigenetic status of Gdnf in the ventral striatum determines susceptibility and adaptation to daily stressful events. *Neuron* 69, 359–372.
- West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J., Takasu, M.A., Tao, X., Greenberg, M.E., 2001. Calcium regulation of neuronal gene expression. *Proc Natl Acad Sci U S A* 98, 11024–11031.
- Yasui, D.H., Peddada, S., Bieda, M.C., Vallerio, R.O., Hogart, A., Nagarajan, R.P., Thatcher, K.N., Farnham, P.J., Lasalle, J.M., 2007. Integrated epigenomic analyses of neuronal MeCP2 reveal a role for long-range interaction with active genes. *Proc Natl Acad Sci U S A* 104, 19416–19421.