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Selective role for DNMT3a in learning and memory

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Abstract

Methylation of cytosine nucleotides is governed by DNA methyltransferases (DNMTs) that establish *de novo* DNA methylation patterns in early embryonic development (e.g., DNMT3a and DNMT3b) or maintain those patterns on hemimethylated DNA in dividing cells (e.g., DNMT1). DNMTs continue to be expressed at high levels in mature neurons, however their impact on neuronal function and behavior are unclear. To address this issue we examined DNMT1 and DNMT3a expression following associative learning. We also generated forebrain specific conditional *Dnmt1* or *Dnmt3a* knockout mice and characterized them in learning and memory paradigms as well as for alterations in long-term potentiation (LTP) and synaptic plasticity. Here, we report that experience in an associative learning task impacts expression of *Dnmt3a*, but not *Dnmt1*, in brain areas that mediate learning of this task. We also found that *Dnmt3a* knockout mice, and not *Dnmt1* knockouts have synaptic alterations as well as learning deficits on several associative and episodic memory tasks. These findings indicate that the *de novo* DNA methylating enzyme DNMT3a in postmitotic neurons is necessary for normal memory formation and its function cannot be substituted by the maintenance DNA methylating enzyme DNMT1.

Keywords

methylation; behavior; long term potentiation; excitatory neurotransmission; fear conditioning

1. Introduction

DNA methylation is a mechanism underlying gene silencing that plays a critical role in establishing and maintaining cellular phenotypes throughout development (Guibert *et al.*, 2009). Methylation of DNA is governed by a family of enzymes, the DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl group to the 5' position of cytosine nucleotides at CG dinucleotides using dietary sources of s-adenosyl-l-

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methionine as the methyl donor. Distinct DNMT proteins establish *de novo* methylation patterns or act to maintain those patterns on unmethylated or hemimethylated DNA. Expression of the maintenance DNA methyltransferase DNMT1 and the *de novo* enzyme DNMT3a is high during development (Szyf *et al.*, 1985) but DNMT mRNA and enzymatic activity are still observed at abundant levels in the adult CNS (Monk *et al.*, 1987; Goto *et al.*, 1994) suggesting the possibility that DNMTs and dynamic methylation states retain functional importance in the adult brain.

Methylation patterns have historically been considered static in postmitotic neurons, however recent work has suggested DNA demethylation at specific promoters may occur contributing to alterations in gene expression that impacts complex behavior and synaptic function (Martinowich *et al.*, 2003; Weaver *et al.*, 2004; Weaver *et al.*, 2005; Weaver *et al.*, 2006; Miller & Sweatt, 2007). In agreement with these findings, treatment with pharmacological DNMT inhibitor compounds has been shown to impair associative learning and inhibit the magnitude and maintenance of long term potentiation (LTP), widely considered a cellular correlate of learning, at hippocampal synapses (Levenson *et al.*, 2006; Miller & Sweatt, 2007; Miller *et al.*, 2008). While there is debate on the mechanism for how DNA demethylation occurs in postmitotic neurons, the potential role of this epigenetic process in cellular functions as well as in neuropsychiatric disorders (e.g., schizophrenia, drug addiction) has attracted a great deal of interest.

To examine the role of DNA methyltransferases in adult brain, mice lacking *Dnmt1* and *Dnmt3a* have been generated. Constitutive *Dnmt1* and *Dnmt3a* knockout mice are not viable, thus research on the role of DNMTs in adult brain function has utilized conditional knockout mice (Li *et al.*, 1992; Okano *et al.*, 1999; Fan *et al.*, 2001; Golshani *et al.*, 2005; Nguyen *et al.*, 2007; Hutnick *et al.*, 2009; Feng *et al.*, 2010; LaPlant *et al.*, 2010). In the current study we investigated the impact of a postnatal, forebrain-specific conditional knockout (CKO) of *Dnmt1* or *Dnmt3a* on complex behavior and synaptic function. Our findings demonstrate clearly dissociable roles for *Dnmt1* and *Dnmt3a* in associative learning tasks as well as in synaptic plasticity. We conclude that *de novo* and maintenance DNMTs cannot functionally compensate for each other in adult brain, and that DNMT3a is critical for normal adult behavior and synaptic function.

2. Materials and Methods

2.1. *Dnmt1* and *Dnmt3a* conditional knockout mice

Floxed *Dnmt1* and *Dnmt3a* lines and the CaMKII α -Cre93 line were on a mixed 129/BALBC background backcrossed to a C57BL/6 line for > 10 generations. Male CaMKII-Cre93 mice were crossed with female floxed homozygous *Dnmt1* or *Dnmt3a* mice, and resulting male Cre-floxed heterozygous *Dnmt1* or *Dnmt3a* were crossed with female floxed homozygous *Dnmt1* or *Dnmt3a* mice to generate conditional KOs (CKOs) of either *Dnmt1* or *Dnmt3a* in forebrain. Littermates derived from these mating paradigms that were Cre-negative served as comparison control mice for the respective groups. Genomic DNA from tail samples was used for genotyping by PCR as previously described (Jackson-Grusby *et al.*, 2001; Nguyen *et al.*, 2007). Mice were tested in the following order in distinct cohorts for the behavioral tasks: cohort 1 – locomotor activity, rotarod, and cued fear conditioning;

cohort 2 – spatial object recognition, novel object recognition, conditioned taste aversion, and contextual fear conditioning. Naïve mice were used for hippocampal slice electrophysiology. All experiments used 6–12 week old male mice maintained on a 12 hr light/dark cycle with *ad libitum* access to food and water except where noted. All experiments were scored by observers blind to the genotype of the mice. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

2.2. Assessment of Dnmt1 and Dnmt3a expression by real-time PCR

Dnmt1, *Dnmt3a*, and *Dnmt3b* expression was examined in adult brain in order to verify the success of our conditional knockout strategy. Bilateral hippocampi, amygdala, prefrontal cortex, and cerebellum were dissected on an ice cold glass dish and placed on dry ice until storage at -80°C . In separate experiments that assessed Dnmt1 and Dnmt3a gene expression in wild type mice after fear conditioning mice were sacrificed 30 minutes post-training. An untrained control group was sacrificed 30 minutes after exposure to the novel context or tone with no exposure to shock. In experiments that assessed gene expression after fear conditioning mice were sacrificed 30 minutes post-training. RNA was extracted from 4–6 animals/group using Trizol reagent and precipitated with isopropanol. After treatment with DNase I to remove residual contaminating genomic DNA, cDNA templates were synthesized using 250 ng RNA with Superscript III reverse transcriptase for 60 minutes at 50°C in the presence of random hexamers. Real-time PCR was performed in triplicate using optimized primers and the following protocol: 95°C for 10 minutes followed by 40 cycles of 95°C for 0.15 seconds and 60°C for 1 minute. Primer sequences used were, 5'-TGG AGA TTA AGC TCT GCC TGC TGT-3' and 5'-TAG TCC TTG GTA GCA GCC TCC TCT TT-3' for *Dnmt1*; 5'-AGT CCC TGC AAT GAC CTC TCC A-3' and 5'-AAC TCA AAG AAG AGG CGG CCA-3' for *Dnmt3a*; 5'-CTG AGA TCT CTG CTG ACA AAC-3' and 5'-TGG TAC ATG GCC TTC CTA TAA-3' for *Dnmt3b*; and the *Gapdh* (see Adachi *et al.*, 2009). β -actin or GAPDH were chosen as housekeeping genes due to similar mRNA expression across all sample templates. Relative gene expression was calculated using the $2^{-\text{ct}}$ method and expressed as fold-induction relative to control (Schmittgen & Livak, 2008).

2.3. Locomotor activity and rotarod testing

Locomotor activity was assessed for 2 hours in a fresh home cage using four photocell beams linked to computer acquisition software (San Diego Instruments). Motor coordination and motor learning were tested by 8 rotarod trials across 2 days (4 trials/day). Mice were placed on a rotating rod (IITC Life Science) which gradually increased speed over the 5-minute trial. Test sessions ended when the animals fell off the rod or after 5 minutes.

2.4. Context- and cue-dependent fear conditioning

Mice were individually tested in operant chambers (Med Associates) with electrifiable stainless steel grid floors and surrounded by a sound-attenuating external chamber. For the training phase of context-dependent fear conditioning, animals were habituated to the chamber [the conditioned stimulus (CS)] for 2 minutes, followed by 3 presentations of

footshock [the unconditioned stimulus (US); 0.5 mA shock, 1 second duration with an inter-shock interval of 1 minute]. Ninety minutes after the training, short-term memory formation was assessed. The mice were placed in the same chambers and behavior was videotaped for 5 minutes. Freezing, defined as no movement except for respiration, was scored at 5 second intervals. For long-term memory formation, the mice were examined 24 hr after the training for a 5 minute time period. Extinction training was carried out for 5 days (i.e., re-exposure to context with no shock). For cue-dependent fear conditioning mice were trained via exposure to 3 tone-shock pairings (30 second white noise tone, 90 dB). Twenty-four hours after training animals underwent extinction training for 5 days in a novel context - a 3 minute baseline period followed by presentations of tone CS alone for 3 minutes. Cue-dependent FC was determined by subtracting baseline freezing from freezing observed during tone.

2.5. Footshock sensitivity and startle amplitude

At the conclusion of the behavioral experiments mice were exposed to increasing footshock intensity (range: 0.05–0.45 mA) to determine the threshold at which the animal responded by vocalization and by jumping. Startle was measured using SR-Lab Startle Response System (San Diego Instruments). Mice were placed into Plexiglass holders and allowed acclimation to the chamber and background white noise (70 dB) for 5 minutes. After acclimation, six startle stimuli (120 dB, 40 ms, white noise) were presented with an average interstimulus interval of 15 seconds (range 7–23 seconds).

2.6. Conditioned taste aversion learning

Mice were adapted to a restricted drinking schedule of two 15-minute drinking sessions per day for 7–10 days. On the training day mice were exposed to a 0.5% saccharin solution for 15 minutes during the morning drinking session rather than the usual presentation of water. Thirty minutes after the onset of saccharin intake animals were injected intraperitoneally (i.p.) with 0.14 M LiCl to induce malaise. A saccharin/water choice-test was given 48 hours after LiCl to determine genotypic effects on acquisition of the aversion. At the conclusion of the experiment animals were given a 0.04 % quinine/water choice test to assess taste sensitivity.

2.7. Spatial and novel object recognition tasks

For spatial object recognition, on day 1 mice were habituated for 10 minutes to an open field chamber (W × L, 39 × 39 cm) decorated with distinct visual stimuli (e.g., solid vertical lines, large star shape). Twenty-four hours later mice were returned to the open field. Mice were given a 10-minute to explore 3 identical objects (e.g., metal tubing) spaced ~8 cm from the walls of the open field. Twenty-four hours later (day 3) the location of 1 object was changed and mice were tested for 6 minutes to determine the ratio of time spent exploring the moved (“spatial”) object to time spent exploring the other 2 objects. The novel object recognition protocol was similar to the spatial object recognition paradigm, however on the test day one object was replaced with a novel object and the ratio of time spent with novel vs. familiar objects was quantified. Animals that did not explore all objects during training and test sessions were excluded. A separate group of animals (n = 5–6/group) were used to determine potential object exploration bias (i.e., whether the animals initially prefer exploring one object versus another). There were no differences in amount of time spent

exploring the different objects used for the experiments (not shown). Spatial and novel object recognition tests and training were carried out in dim lighting.

2.8. Hippocampal slice recordings

Hippocampal slices were prepared as described previously (Morris *et al.*, 2013). Input-output relationship was determined by providing an ascending series of stimulus input intensities (range 40 – ~240 μ A) until the maximum field excitatory postsynaptic potential (fEPSP) response was determined. An input stimulus intensity that induced 40–50% of the maximum response was used for measuring paired-pulse ratio (PPR) and theta burst-induced LTP. An input intensity that induced ~75% of the maximum response was used for high frequency stimulation (HFS)–induced LTP. PPR was induced by giving 2 pulses at decreasing interpulse intervals (400, 200, 100, 50, 30, and 20 ms) and analyzed by dividing the fEPSP slope of pulse 2 by pulse 1. Following 20 minutes of stable baseline fEPSP slope, LTP was induced by theta burst (3 trains with 3 100 Hz bursts/train. Each burst consisted of 5 pulses with an interburst interval of 200 ms and intertrain interval of 10 s), or HFS (4 trains of 100 pulses/train at 100 Hz, inter-train interval of 20 seconds).

2.9. Statistical analysis

Data were analyzed by Student's *t*-tests, two-way ANOVA with repeated measures, or one-way ANOVA, with post-hoc Fischer's LSD tests conducted following significant interaction effects. Input-output slopes from hippocampal field recordings were fit by linear regression and statistical significance of slope differences was determined by *t*-test. Slopes that did not achieve a fit of $r^2 > 0.80$ were discarded. Data are presented as mean \pm SEM. All analyses were two-tailed with *p*-value ≤ 0.05 considered statistically significant.

3. Results

3.1 Fear conditioning increases Dnmt3a expression in brain

We examined *Dnmt1* and *Dnmt3a* expression in C57BL/6 mice following training in a fear conditioning task, using quantitative PCR (qPCR) on tissue from brain regions important for associative fear learning. *Dnmt1* mRNA was unchanged in hippocampus, frontal cortex or amygdala following training in cued or contextual fear conditioning (Fig. 1A, B). In contrast, *Dnmt3a* mRNA was rapidly (30 minutes) increased in the amygdala, a brain region necessary for associative fear learning, following training in cued fear conditioning (Fig. 1A, B). At this same time point following training in contextual fear conditioning, *Dnmt3a* was significantly increased in the hippocampus, which is required for contextual, not cued, fear learning (Kim & Fanselow, 1992; Kim & Jung, 2006).

3.2 Dnmt3a CKO mice exhibit associative learning deficits

Given the dissociable effects of fear conditioning on *Dnmt* expression we hypothesized that DNMT1 and DNMT3a are uniquely involved in learning this task, which requires animals to associate a previously innocuous conditioned stimulus (CS) with a salient unconditioned stimulus (US). To test this hypothesis conditional *Dnmt1* or *Dnmt3a* knockout mice were generated using the calcium-calmodulin-dependent protein kinase II promoter (CaMKII α) to drive expression of Cre recombinase (CaMKII α -Cre93 line) to delete *Dnmt1* or *Dnmt3a* in

forebrain neurons starting at approximately two weeks of age (Chen *et al.*, 2001). This conditional knockout (CKO) strategy reduced *Dnmt1* or *Dnmt3a* mRNA expression ~70–85% in adult forebrain regions including the hippocampus, frontal cortex and amygdala without affecting expression in the cerebellum or compensating other *Dnmt* gene expression (Fig. 1C, D, E). The *Dnmt1* or *Dnmt3a* knockout mice also had indistinguishable body weight, brain weight, locomotor activity, and motor learning relative to littermate control (CTL) mice (Fig. 2A–G).

Dnmt1 CKO mice performed similarly to CTL in contextual and cued fear conditioning (Fig. 3A, C). By contrast, *Dnmt3a* CKO mice had impaired memory formation 24 hours post-training along with abnormal extinction 72 hours after training in contextual fear conditioning (Fig. 3B). In a cued fear learning task persistent deficits in the ability to extinguish the tone-footshock association manifest 3 days post-training and persisted for the remainder of testing (Fig. 3D), however long term memory tested 24 hours after training did not differ from CTL mice. *Dnmt3a* CKOs had normal nociception, startle responses and baseline freezing (not shown) ruling out differential pain sensitivity, audition, or freezing in a novel environment as confounding factors in our study. A separate cohort of mice was used to test short-term fear learning. *Dnmt1* and *Dnmt3a* CKO mice did not exhibit any differences compared with CTL mice in context fear conditioning tested 90 minutes after training (data not shown). To further investigate a specific role for DNMT3a in associative learning, we examined CKO mice in a conditioned taste aversion (CTA) paradigm, in which a novel taste CS is paired with an illness inducing unconditioned stimulus and results in subsequent avoidance of the novel taste. Again *Dnmt1* CKO mice learned normally, while *Dnmt3a* CKOs displayed less of an aversion to 0.5% saccharin solution compared with CTL mice 48 hours after this novel taste CS was paired with 0.14M LiCl, a US which induces malaise (Fig. 3E–G). CTA learning relies on brain circuits distinct from those involved in fear learning (Yamamoto *et al.*, 1994), suggesting a general involvement of DNMT3a in associative learning.

To determine if the effects of *Dnmt3a* CKO were specific for associative learning, we tested spatial and novel object recognition, in which normal mice spend more time exploring objects that have been displaced from their original spatial location or from novel objects. *Dnmt3a* CKO mice failed to form memories for spatial object location or for the nature of the object itself, as demonstrated by no preference for relocated or novel objects, whereas *Dnmt1* CKO mice displayed no deficits (Fig. 3H, I).

3.3 Long-term potentiation in hippocampal CA1 is deficient in *Dnmt3a* CKO mice

Long-term potentiation (LTP), a putative cellular mechanism underlying learning, was examined using theta burst stimulation (TBS)- a physiologically relevant stimulus that mimics *in vivo* hippocampal firing during learning (Otto *et al.*, 1991). Field recordings from hippocampal slices from *Dnmt1* CKO mice revealed normal TBS-induced LTP in the CA1 subregion of HC (Fig. 4A). In contrast, *Dnmt3a* CKO slices had impairments in the induction and maintenance of LTP compared to slices from CTL mice (Fig. 4B). We next used 4-train high frequency stimulation (HFS), a robust stimulus that, unlike the theta burst protocol, produces long-lasting LTP. We found that although the LTP magnitude was not

significantly different from CTL for ~40 minutes after HFS, deficits in LTP magnitude subsequently manifest in *Dnmt3a* CKO slices and LTP was not maintained (Fig. 4C). Basal synaptic function as well as short-term plasticity appeared largely normal in *Dnmt1* and *Dnmt3a* CKOs as demonstrated by input-output relationships and paired pulse ratio (PPR) (Fig. 4D–G), suggesting the deficits were specific for long-term synaptic plasticity. *Dnmt1* CKO exhibited enhanced PPR relative to CTL at one of six inter-stimulus intervals tested, suggesting the possibility of mild neurotransmitter release deficits that do not significantly impact the learning and memory behaviors we assessed (Fig. 4F).

4. Discussion

In the current study we report a critical role for DNMT3a in complex behavior and synaptic function in the adult CNS. Our studies demonstrate that experience in an associative fear learning task impacts expression of *Dnmt3a*, but not *Dnmt1*, in brain areas that mediate learning of this task. To further explore this finding, we examined conditional *Dnmt3* KO mice and observed impairments in learning and memory as assessed by several tasks that assay distinct forms of learning and memory. *Dnmt3a* CKO mice had impairments in spatial displaced object or novel object recognition. Associative memory formation and extinction of previously learned associations were also deficient in *Dnmt3a* CKOs as evidenced by impaired performance relative to CTL mice in both contextual and cued fear conditioning and a CTA paradigm. Conversely, in all cases of learning and memory task performance, we found no phenotypes in *Dnmt1* CKO mice.

The hippocampal electrophysiological phenotypes promoted by *Dnmt3a* or *Dnmt1* CKO were also dissociable. The impact of *Dnmt1* CKO on electrophysiological properties in the CA1 subregion of hippocampal slices was relatively mild as we found only an increase in PPF at a short (30 ms) interpulse interval and no changes in input-output functions. Furthermore, LTP was normal in *Dnmt1* CKOs, in contrast to *Dnmt3a* CKOs that were impaired using a theta burst stimulus or more robust high frequency stimulation. These data are consistent with the behavioral results in which we found no impact of *Dnmt1* CKO on learning whereas we observed learning deficits in the *Dnmt3a* CKOs.

The present study provides evidence for dissociable roles for DNMT1 and DNMT3a in associative learning, extinction learning, and synaptic plasticity. These data are consistent with previous work showing DNMT3a manipulation in forebrain can induce robust behavioral phenotypes (LaPlant *et al.*, 2010; Oliveira *et al.*, 2012). Experience in a fear learning task or chronic treatment with drugs of abuse has been shown to alter the expression of *Dnmt3a*, and not *Dnmt1*, in the forebrain, an effect consistent with a distinct role for these enzymes in adult behavior (Miller & Sweatt, 2007; LaPlant *et al.*, 2010). Furthermore, a recent study found decreased *Dnmt3a* expression in the brains of aged mice that correlated with poor performance on tests of memory, while restoration of the level of *Dnmt3a2*, one of two transcripts from the *Dnmt3a* gene, improved memory in fear conditioning and spatial object recognition (Oliveira *et al.*, 2012).

Our findings are seemingly in contrast to a recent study that reported deletion of *Dnmt1* or *Dnmt3a* in forebrain, using the same CaMKII α -Cre93 line, did not impact learning although

a concurrent knockout of both *Dnmt1* and *Dnmt3a*, not tested in our study, produced learning and memory as well as synaptic plasticity deficits (Feng *et al.*, 2010). In the previous study, learning and memory was assessed by the Morris water maze and by contextual fear conditioning 24 hours after training. In the current study, we sought to examine the conditional *Dnmt1* and *Dnmt3a* CKOs in several other learning and memory paradigms. In the fear conditioning paradigm, we observed a significant deficit in context dependent learning of the *Dnmt3a* CKO mice at 24 hours after training as well as in rates of extinction. The *Dnmt3a* CKO mice in cue dependent fear conditioning had significant differences in extinction of the cue starting at 72 hours after training that persisted for at least 120 hours. We also examined the individual *Dnmt1* and *Dnmt3a* CKOs in novel and spatial objection recognition as well as conditioned taste aversion where we noticed specific deficits in learning associated with the loss of *Dnmt3a* and not *Dnmt1*. These data suggest that DNMT3a is involved in specific types of learning and memory distinct from DNMT1. The reason for contrasting behavioral results between our current study and Feng *et al.* (2010) are not clear, however it is possible that gender and/ or age differences between mice used in their experiments and ours may have contributed. Our study used only male mice between 6–12 weeks of age, whereas their study included both males and females and the age of the mice was not reported. We also examined our individual *Dnmt1* and *Dnmt3a* CKOs for alterations in LTP as well as paired pulse facilitation. Consistent with our results the study by Feng reported no deficits in LTP in the single *Dnmt1* CKO mice using a tetanic stimulation and also did not find alterations in prepulse facilitation or in analysis of input-output curves. In *Dnmt3a* CKO slices we observed significant deficits in the induction and maintenance of LTP in the *Dnmt3a* CKOs using theta burst stimulation and a high frequency stimulation, however we did not observe any changes in paired pulse facilitation or in the analysis of input-output curves. The Feng *et al* study stated that the single knockout mice showed no deficit in LTP, however data was not shown regarding the analysis of LTP in the *Dnmt3a* single knockout mice leaving it difficult to make a direct comparison between the two studies. The consistent findings that loss of DNMT1 or DNMT3a do not impact paired pulse facilitation or basal synaptic transmission as assessed by input-output curves suggests that DNMT3a impacts specific aspects of synaptic plasticity and not global aspects.

The mechanisms responsible for increased *Dnmt3a* expression following learning in the fear-conditioning paradigm are unknown. However, taken together with our findings that *Dnmt3a* CKO mice have learning and synaptic deficits, it is possible that the upregulation of *Dnmt3a* triggers *de novo* DNA methylation on genes involved in these processes while the deficits observed in the *Dnmt3a* KO mice are the result of the loss of this mechanism. This hypothesis would be in agreement with recent *in vitro* work demonstrating that NMDA receptor mediated synaptic activity drives DNA demethylation in hippocampal neurons (Nelson *et al.*, 2008). Collectively, these data suggest an important and critical role for transcriptional repression in learning and synaptic function (Monteggia & Kavalali, 2009).

Our findings that fear conditioning regulated *Dnmt3a* expression and that loss of *Dnmt3a* resulted in learning and synaptic deficits support the growing literature for an importance of epigenetic mechanisms in the adult brain. The gene targets that may be ultimately impacted in learning and memory by *Dnmt3a* are currently unknown. Previous work has demonstrated

that experience in appetitive or aversive learning tasks impacts the methylation of various genes with known involvement in memory formation including *BDNF*, *protein phosphatase-1*, *reelin*, *calcineurin*, and the immediate early genes *Erg1* and *c-fos* (Miller & Sweatt, 2007; Lubin *et al.*, 2008; Miller *et al.*, 2010; Mizuno *et al.*, 2012; Day *et al.*, 2013). The further identification and characterization of putative targets will be important in deciphering the role of DNA methylation changes in learning and memory, as little is known about this process in the adult central nervous system. Taken together, our data suggest that *de novo* and maintenance DNMTs play functionally distinct roles in the adult brain in learning and memory.

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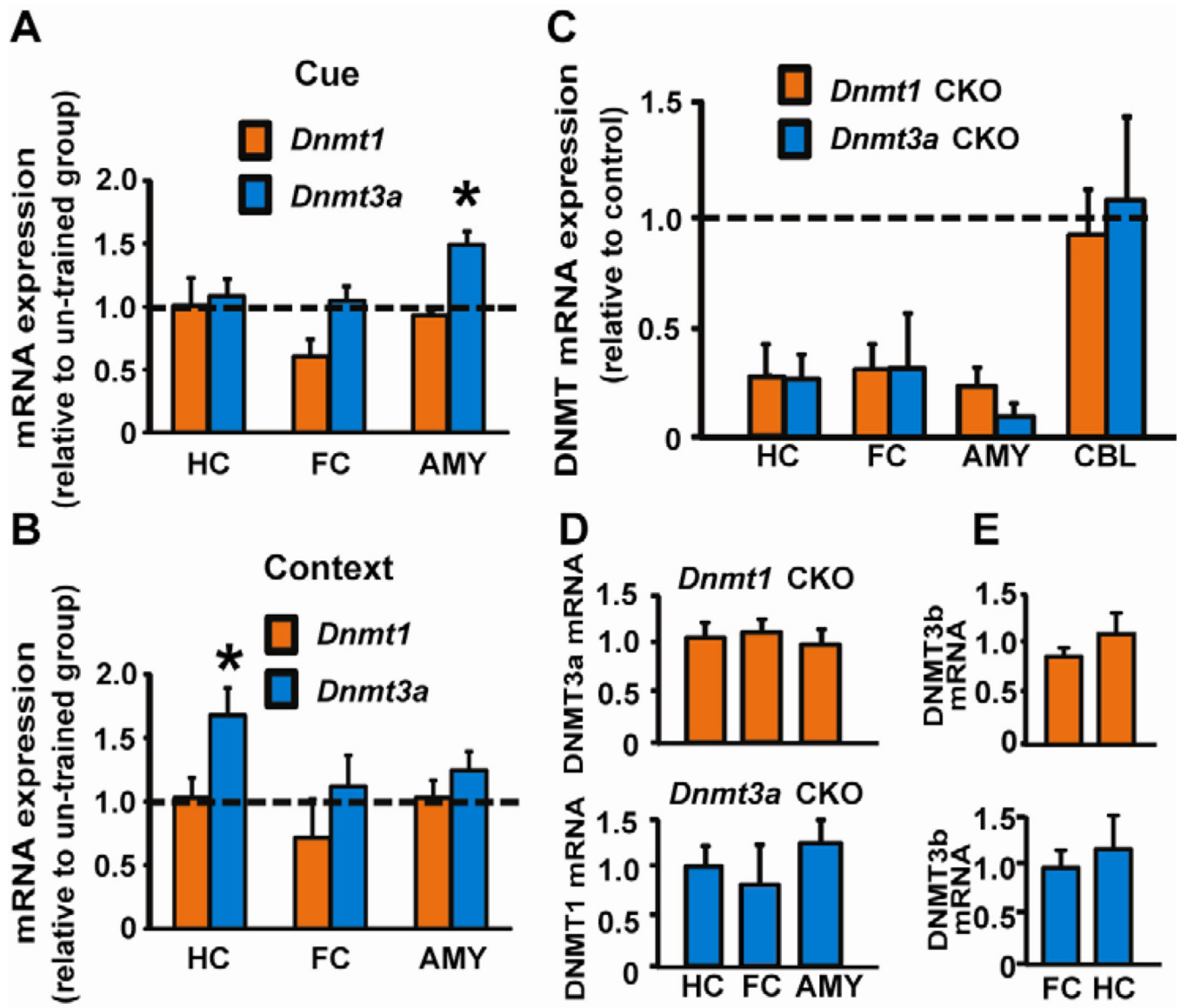


Fig. 1.

Dnmt1 and *Dnmt3a* mRNA expression in mouse brain. (A) After training in cued fear conditioning *Dnmt3a* expression was increased in the amygdala (AMY) of C57BL/6 mice relative to un-trained mice [$t(8) = 3.219$, $p = 0.012$]. A trend ($p = 0.062$) for reduced *Dnmt1* expression was evident in frontal cortex (FC). (B) Training in contextual fear conditioning increased *Dnmt3a* expression in hippocampus (HC) [$t(10) = 2.30$, $p = 0.049$] with a trend in AMY ($p = 0.16$); *Dnmt1* expression was unchanged in HC, FC, and AMY. (C) Consistent with forebrain-specific deletion, *Dnmt1* or *Dnmt3a* mRNA was reduced in 6-week old CKOs in HC, AMY, and FC, with no change in cerebellum (CBL). (D) No compensation in *Dnmt3a* expression when *Dnmt1* was knocked down (top panel), and vice versa (bottom panel). (E) No changes in *Dnmt3b* expression in *Dnmt1* and *Dnmt3a* CKO mice (top and bottom panels, respectively) in comparison to control animals. * $p < 0.05$ compared with un-trained CTL ($n = 4-6$ /group in all groups).

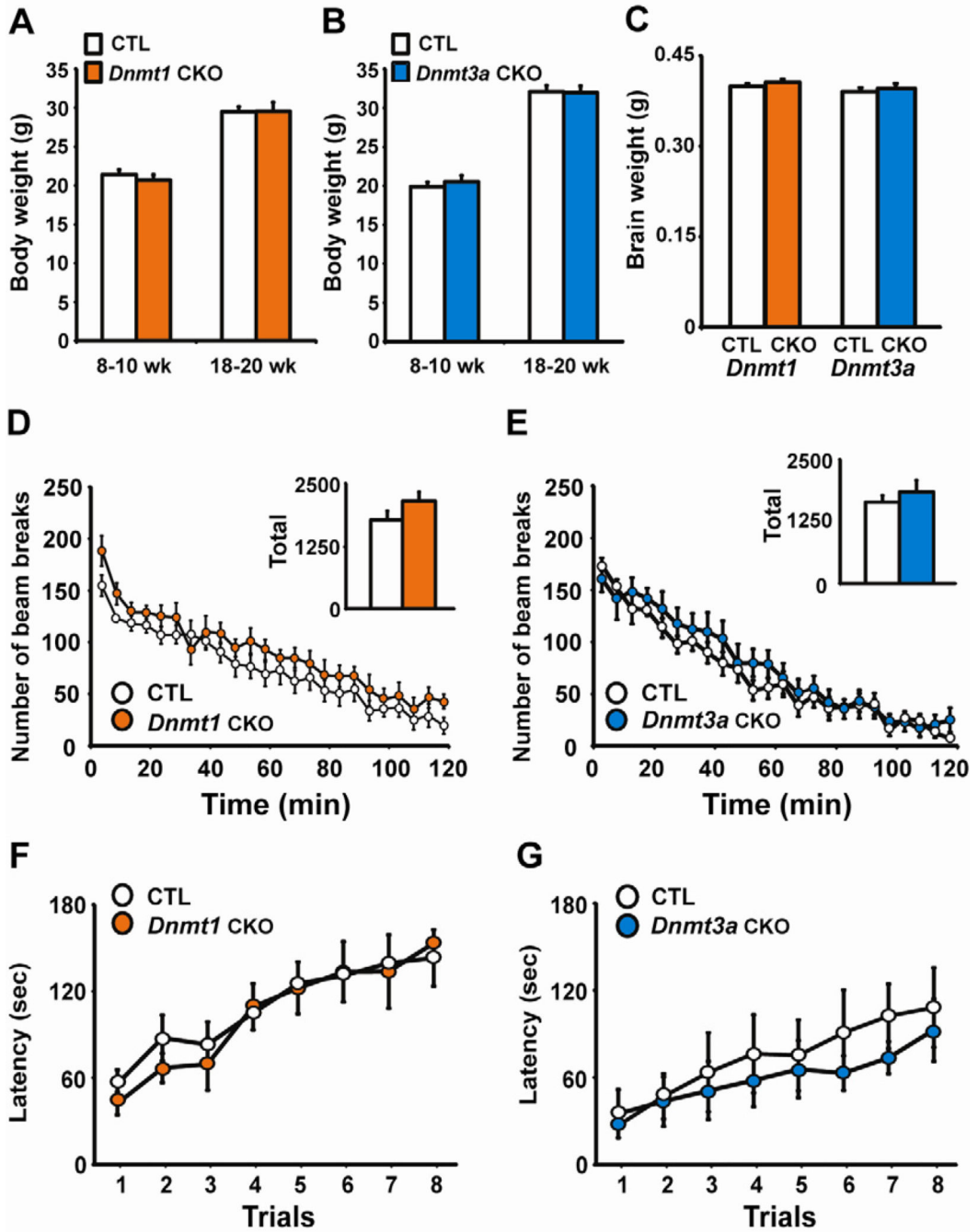


Fig. 2. Normal body and brain weights and motor behavior in *Dnmt1* and *Dnmt3a* CKO mice. (A–C) *Dnmt1* or *Dnmt3a* CKO did not adversely affect body weight or brain weight measured at 10 weeks, n=10–12/group. (D–E) Locomotor activity was normal in *Dnmt1* (n=8) and *Dnmt3a* (n=10) CKO mice relative to control littermates (CTL, n=8, 10). Inset displays total locomotion over the 2-hour test. (F–G) *Dnmt1* (n=8) and *Dnmt3a* (n=10) CKOs had normal motor coordination and normal motor learning relative to CTL (n=8, 10) as demonstrated by performance on the rotarod task.

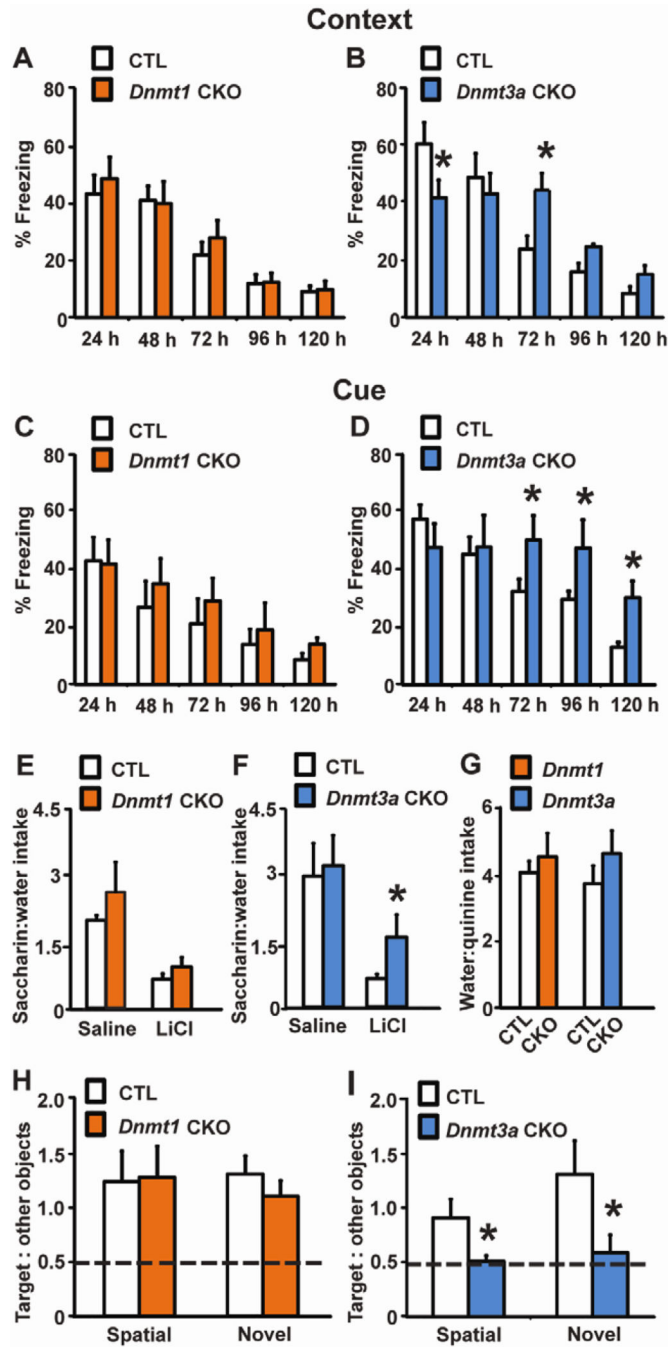


Fig. 3. *Dnmt3a* CKOs have learning and memory deficits. (A–B) In context-dependent fear conditioning *Dnmt1* (n=10) CKOs have normal learning (CTL, n=12), while *Dnmt3a* (n=10) CKOs exhibited deficits in long-term memory 24 hours after training [$t(16) = 2.158, p = 0.047$] and extinction 72 hours post-training [$t(16) = 2.633, p = 0.018$] compared to CTL (n=8). (C–D) in cue-dependent fear conditioning, *Dnmt1* (n=12) CKOs learned normally, while *Dnmt3a* (n=8) CKOs had impaired extinction relative to CTL (n=12, 8) [72 hours post-training: $t(14) = 2.140, p = 0.05$; 96 hours : $t(14) = 2.166, p = 0.049$; 120 hours : $t(14) =$

3.011, $p = 0.009$]. (E–G) Saline-treated *Dnmt1* ($n=11$) and *Dnmt3a* ($n=8$) CKOs had normal 0.5% saccharin preference compared to CTL ($n=11$, 10). Forty-eight hours after training LiCl-treated *Dnmt1* ($n=11$) CKOs showed normal acquisition of an induced taste aversion to saccharin, however *Dnmt3a* ($n=9$) CKOs exhibited deficits in memory as evidenced by increased saccharin:water intake ratios relative to LiCl-treated CTL mice ($t(14) = 2.167$, $p = 0.048$). (G) Deficits in CTA learning could not be explained by genotypic differences in taste sensitivity as both CKOs preferred water to 0.04% quinine equally to CTL. (H) *Dnmt1* ($n=8$) CKOs had normal spatial or novel object recognition relative to CTL ($n=11$). (I) *Dnmt3a* CKOs had severe deficits and performed near chance level (dashed line) for both spatial object recognition and novel object recognition [spatial, $t(18) = 2.226$, $p = 0.039$; novel, $t(14) = 2.275$, $p = 0.038$]; *Dnmt3a* CKO, spatial object recognition $n=10$, novel object recognition $n= 8$; CTL, spatial object recognition $n=10$, novel object recognition $n= 8$). * $p < 0.05$ vs. CTL

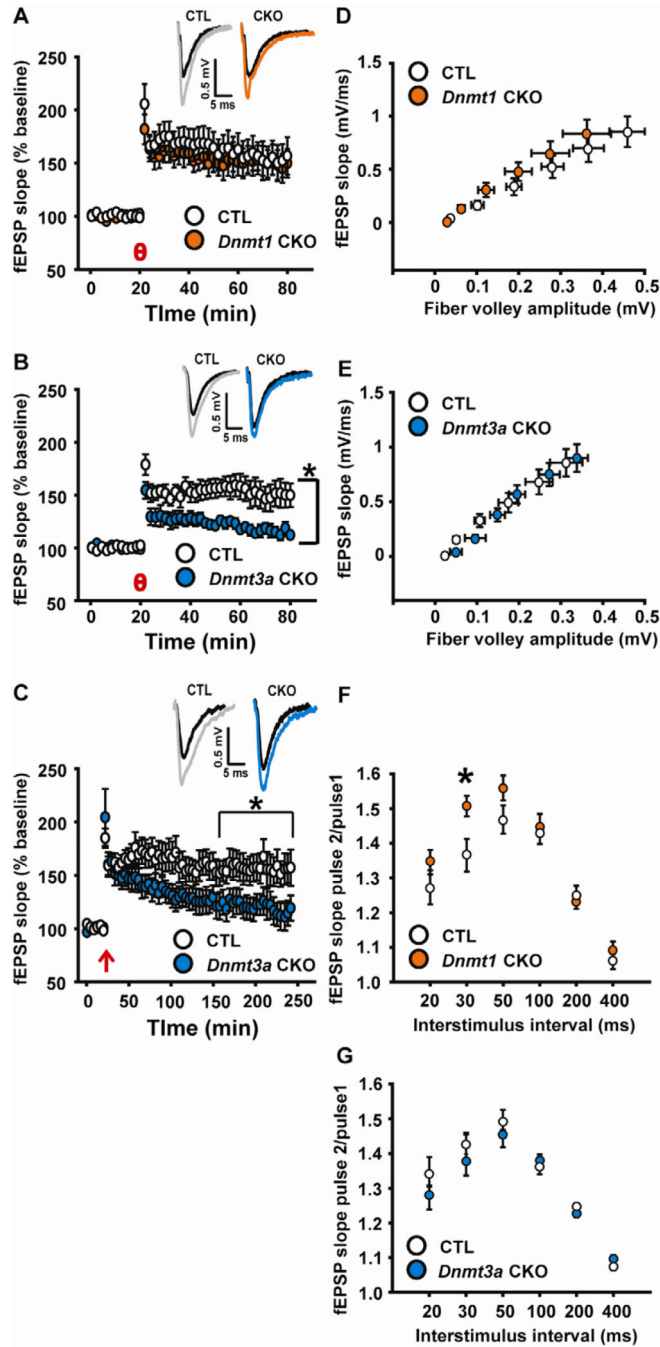


Fig. 4. Dissociable effects of *Dnmt1* and *Dnmt3a* CKO on LTP in hippocampal CA1. (A–B) Hippocampal slices prepared from *Dnmt1* CKO mice (n=10) had normal theta burst-induced LTP relative to CTL (n=9), however *Dnmt3a* CKO slices had deficits in LTP following theta burst (time × genotype interaction $F_{(159, 3517)} = 4.006$, $p < 0.001$, 90.0 % of points after theta burst $p < 0.05$). (C) *Dnmt3a* CKO mice were also impaired following high-frequency stimulation (time × genotype interaction $F_{(427, 6213)} = 3.392$, $p < 0.001$, 68.77% of points after high frequency stimulation $p < 0.05$; 100% of points significant 157 minutes after LTP

induction until end of recording) relative to CTL (*Dnmt3a* CKO, theta burst n=10, HFS n=6; CTL, theta burst n=12, HFS n=7). θ = theta burst; \uparrow = high frequency stimulation. Insets (A–C) - Representative traces from CTL and *Dnmt1* or *Dnmt3a* CKO slices 30 (A, B) or 120 minutes (C) after LTP induction. Black traces = baseline, gray (CTL), or colored traces are after LTP induction. (D, E) Input-output relationships were normal in both *Dnmt1* (n=11) and *Dnmt3a* (n=6) CKOs relative to control littermate (CTL, n=9, 8) hippocampal slices. (F, G) *Dnmt1* (n=10) CKOs exhibited significantly enhanced paired pulse ratio at 30 ms interstimulus interval ($t(15) = 2.346$, $p = 0.033$), suggesting decreased neurotransmitter release probability compared with CTL (n=7). *Dnmt3a* (n=6) CKO mice had paired pulse ratios that did not differ from CTL (n=8). * $p < 0.05$ vs. CTL.