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Tet1 oxidase regulates neuronal gene transcription, active DNA hydroxymethylation, object location memory, and threat recognition memory

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ABSTRACT

A dynamic equilibrium between DNA methylation and demethylation of neuronal activity-regulated genes is crucial for memory processes. However, the mechanisms underlying this equilibrium remain elusive. Tet1 oxidase has been shown to play a key role in the active DNA demethylation in the central nervous system. In this study, we used *Tet1* gene knockout (Tet1KO) mice to examine the involvement of Tet1 in memory consolidation and storage in the adult brain. We found that *Tet1* ablation leads to altered expression of numerous neuronal activity-regulated genes, compensatory upregulation of active demethylation pathway genes, and upregulation of various epigenetic modifiers. Moreover, Tet1KO mice showed an enhancement in the consolidation and storage of threat recognition (cued and contextual fear conditioning) and object location memories. We conclude that Tet1 plays a critical role in regulating neuronal transcription and in maintaining the epigenetic state of the brain associated with memory consolidation and storage.

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1. Introduction

Recent findings have clearly implicated a role for DNA methylation in memory formation and storage (Miller and Sweatt, 2007; Lubin et al., 2008; Feng et al., 2010a; Miller et al., 2010; Lesburgueres et al., 2011; Monsey et al., 2011; Sweatt, 2013). Conventionally, DNA methylation has been considered to act as a transcriptional silencer (Jaenisch and Bird, 2003; Bonasio et al., 2010; Feng et al., 2010b). However, recent studies point toward a more complex role of DNA methylation based on the cell type involved or the genomic context in which the methylation event occurs (Yu et al., 2013; Bahar Halpern et al., 2014; Jeltsch and Jurkowska, 2014). Also, recent discoveries have identified the presence of DNA methylation at "unconventional" non-CpG (cytosine-guanine dinucleotide sequences) sites (Xie et al., 2012; Lister et al., 2013; Varley et al., 2013). Until recently, DNA methylation was considered to be essentially irreversible; however, new discoveries have shown that the methylation of memory-associated genes can be dynamic and reversible, strongly indicating the presence of an active DNA demethylation pathway in the adult brain (Miller and Sweatt, 2007; Lubin et al., 2008; Ma et al., 2009; Guo et al., 2011). The concurrent discovery of the ten-eleven translocation (Tet) family of proteins (Tahiliani et al., 2009) and the rediscovery of the 5hmC base in DNA derived from the central nervous system (CNS) (Penn et al., 1972; Kriaucionis and Heintz, 2009) suggested the presence of a TET-driven active DNA demethylation pathway in the brain.

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Tet proteins (TET1, 2, and 3) were identified as Fe(II)- and 2-oxoglutarate–dependent dioxygenases that can oxidize 5mC to 5hmC (Iyer et al., 2009; Kriaucionis and Heintz, 2009; Tahiliani et al., 2009; Globisch et al., 2010), and recent studies have given some insight into the role of Tet1 and Tet3 as drivers of active cytosine demethylation in the CNS (Guo et al., 2011; Kaas et al., 2013; Rudenko et al., 2013; Zhang et al., 2013; Li et al., 2014). Tet1KO mice have been shown to be developmentally normal (Dawlaty et al., 2011) and to manifest robust fear conditioning memory refractory to memory extinction (Rudenko et al., 2013). Recently, our laboratory showed that Tet1 expression exhibited a learning-associated downregulation in vivo, and the overexpression of TET1 in adult dorsal hippocampus leads to a deficit in long-term fear conditioning memory (Kaas et al., 2013). These findings suggested that Tet1 might act as a negative memory regulator; therefore, in the present studies, we extended the findings of Kaas et al. and tested the hypothesis that Tet1 deletion might improve memory acquisition, consolidation, and storage in contextual and cued Pavlovian threat (fear) conditioning paradigms.

We, for the first time to our knowledge, report that Tet1KO mice have enhanced threat recognition (also known as *fear conditioning*), (Ledoux, 2014) memory consolidation and storage. We also found that a virally mediated shRNA knockdown of *Tet1* in dorsal hippocampus led to an enhanced long-term memory for object location. Moreover, *Tet1* gene ablation led to alterations in various neuronal activity–regulated genes, including key genes from the cyclic adenosine monophosphate (cAMP) transcription-regulating pathway that have previously been shown to be critical in long-term memory consolidation. We also observed that *Tet1*-deficient animals exhibit upregulation of genes related to epigenomic modifications and DNA demethylation pathway including *Tet2* and *Tet3*.

Overall, we show in this study that Tet1 is crucial in maintaining the methylation status of the brain by controlling 5hmC production and that it is also critical for the regulation of neuronal gene transcription. Most importantly, deletion of TET1 led to enhanced memory consolidation and storage. Therefore, Tet1 inhibition might serve as a useful pharmacologic target for cognitive enhancement.

2. Material and methods

2.1. Animals and genotyping

Tet1+/− mice were purchased from The Jackson Laboratory (strain name: B6;129S4-Tet1tm1.1Jae/J, stock no.: 017358) and were bred as heterozygotes at the University of Alabama at Birmingham. Wild-type (WT) and knockout (KO) male offspring of the heterozygote parents of age between 3 and 4 months were used for all the behavior and molecular experiments. For the object location memory (OLM) task, 10–12-week-old C57BL/6 male mice from Harlan were injected rAAVs through stereotaxic surgeries. Animals were singly housed 3 days before the start of any behavior experiments. Animals were maintained under a 12-hour light/dark schedule with access to food and water ad libitum. All studies were performed in compliance with the University of Alabama Institutional Animal Care and Use Committee guidelines.

2.2. Cresyl violet staining

Tet1KO and WT males of age between 3 and 4 months were used for sectioning. Protocol for cresyl violet staining was adopted from Almonte et al. (2013).

2.3. Quantitation of global modified cytosines using mass spectrometry

Extraction, hydrolysis, and quantification of the cytosines and modified cytosines were done according to the method described previously in Kaas et al. (2013). Tet1KO and WT (n = 4 males per group) mice of age between 3 and 4 months were used for the tissue extraction. Statistical comparisons between 2 groups were performed using an unpaired *t* test (2 tailed). Statistical analysis between 3 or more groups was accomplished using 1-way analysis of variance (ANOVA) with Bonferroni post hoc test.

2.4. Behavior tasks

Baseline behavior assessments were performed as previously described (Chwang et al., 2007). Social approach paradigm was adopted from Silverman et al. (2010) and Ellegood et al. (2013). Regarding our nomenclature for Pavlovian associative conditioning to aversive foot-shock stimuli, in this article, we refer to that form of learning as *threat recognition training*. The protocols we used for threat recognition training are identical to those we have used previously and have referred to in prior publications as *fear conditioning* (see, e.g., Chwang et al., 2007). However, beginning with this manuscript, we have updated our nomenclature to more accurately reflect what is likely occurring with foot-shock conditioning, that is, threat response conditioning and not *fear* conditioning per se. The rationale behind this change in nomenclature was recently and clearly articulated by Joe LeDoux, and we fully ascribe to this new viewpoint (Ledoux, 2014). For the threat recognition training (fear conditioning) paradigm, animals were first trained for a total of 3 minutes in a novel context. The first 2 minutes was given as habituation period; after that, an audio cue, 75 dB, was played for 30 seconds, immediately followed by a foot shock (0.5 mA, 1 second, for "light" and 0.8 mA, 2 seconds, for the "strong" training). The animals were given an additional 30 seconds in the cage before removal. For the robust training paradigm, animals received 3 tone-shock pairings (75 dB, 0.8 mA, 2 seconds) every 1 minute after the first 2 minutes of habituation, and the animal was removed after a total of 6½ minutes. For contextual testing, animals were placed back in the same context for 3 minutes after 1 hour of training for testing short-term memory and after 24 hours, 15 days, and 30 days of training for assessing long-term memory storage. For cued testing, animals were placed in a modified context for 5 minutes, and the same audio cue was presented during the last 3 minutes. Percent freezing was scored manually with the evaluator blinded to genotype. Tet1KO and WT males (n = 8 males per group) of the age between 3 and 4 months were used for all the behavior studies. Statistical comparisons between 2 groups were performed using an unpaired *t* test (2 tailed). Statistical analysis between 3 or more groups was accomplished using one-way ANOVA with Bonferroni post hoc test.

2.5. OLM task

The OLM task was adapted from Stefanko et al. (2009) and Haettig et al. (2011). Both the OLM training and the testing sessions were recorded using TopScan (Clever Sys, Reston, VA), and the data were analyzed by the individual blinded to the genotype of each animal. The relative exploration time was recorded and expressed as a discrimination index (D.I. = [t-novel − t-familiar]/[t-novel + t-familiar] × 100). C57BL/6 mice from Harlan, 10–12 weeks of age (n = 8 males per group), were used. Statistical comparisons between 2 groups were performed using an unpaired *t* test (2 tailed).

2.6. rAAVs and stereotaxic surgeries

High titers of recombinant AAV2/9 virus expressing either a hemagglutinin-tagged human TET1 catalytic or a catalytically inactive TET1 mutant (H1671Y/D1673A) domain were generated as previously described (Guo et al., 2011). Viral injection into the dorsal hippocampus of 10–12-week-old C57BL/6 mice was done using the following stereotaxic coordinates: −2 mm anteroposterior, ±1.5 mm mediolateral, and −1.6 mm dorsoventral from bregma. A total of 1.5 μL of viral solution per hemisphere was injected. Injections were

performed using a 10-mL Hamilton Gastight syringe controlled by a Pump 11 Elite Nanomite Programmable Syringe Pump (Harvard Apparatus). The injections proceeded at a speed of 150 nL min^{-1} through a 32-gauge needle. The injection needle was left in place for an additional 5 minutes to allow the fluid to diffuse. Behavioral experiments were performed 30 days following stereotaxic delivery of rAAVs. Electrophysiology experiments were conducted 14 days following AAV-eYFP, Tet1, or Tet1m viral injection.

2.7. Tissue collection and gene expression

Hippocampal subdissections were done as described in Lein et al. (2004). In the case of cortex, predominantly prefrontal cortex (plus some adjacent tissue) was taken for all molecular analyses. All dissections were carried out under a dissecting scope and immediately frozen on dry ice and stored at −80°C until further processing. RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. One hundred fifty nanograms of total RNA was converted to cDNA using the iScript cDNA synthesis Kit (Bio-Rad). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on an iQ5 RT-PCR detection system using iQ SYBR Green Supermix and 300 mol/L of primer. All qRT-PCR primers were designed using Primer Quest (Integrated DNA Technologies) to span exon-exon junctions or were acquired directly as predesigned PrimeTime qPCR Primer Assays (Integrated DNA Technologies). For all qRT-PCRs, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used an internal control. The gene expression analysis was done using the comparative Ct method adopted from Livak and Schmittgen (2001) and Pfaffl (2001). An R package, ComplexHeatmap (https://github.com/jokergoo/ComplexHeatmap), was used to make heat maps from the qRT-PCR gene expression data. Statistical comparisons between 2 groups were performed using an unpaired *t* test (2 tailed).

2.8. Electrophysiology

Extracellular field potential recordings from hippocampal area CA1 Schaffer collateral synapses were obtained as described earlier (Feng et al., 2010a). Baseline synaptic transmission was plotted using stimulus intensities between 1 and 30 mV and evoked field excitatory postsynaptic potential (EPSP) slopes. Subsequent experimental stimuli were set to an intensity that evoked an fEPSP that had a slope of 50% or 25% of the maximum fEPSP slope. Various time intervals (10–300 milliseconds) between constant-stimulus paired pulses were used to measure paired-pulse facilitation (PPF). For inducing long-term potentiation (LTP), 3 different stimuli were used: in the first 2 cases, LTP was induced using 1 tetanus stimulus of 100 Hz for either 1 or 0.1 second; and in the third case, LTP was induced using a 1-time theta burst stimulation (1 episode of theta burst stimulation, 10 bursts at 5 Hz, each with 4 stimuli at 100 Hz with 20-second intervals). Data were recorded for 3 hours following stimulation. Field EPSPs were recorded every 20 seconds (traces were averaged for every 2-minute interval). Animals (n = 6 males per group, 7 slices each) of age 3–4 months were used for making slices. Analysis was done using an unpaired *t* test (2 tailed) and 2-way ANOVA.

2.9. Statistical analysis

All statistical analysis was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Tet1 deletion does not affect overall adult brain morphology

Tet1KO mice were originally generated by deletion of exon 4 and were shown to be grossly normal (Dawlaty et al., 2011; Rudenko et al., 2013). To check for any morphological defects in the brain, we used cresyl violet staining of brain sections of the WT and KO mice. Tet1KO mice showed no obvious morphological differences in comparison to WT (Fig. 1A–C). The loss of *Tet1* mRNA in Tet1KO animals was confirmed by qRT-PCR ($^{****}P < .0001$, Fig. 1D).

3.2. 5hmC is enriched in brain areas involved in active memory processing

The exact function of 5hmC is not yet known. However, relative 5hmC levels, but not 5mC levels, have been consistently shown to be highest in the brain compared with all other tissues (Kriaucionis and Heintz, 2009; Globisch et al., 2010; Munzel et al., 2010). This implies that 5hmC may have important brain-specific functions. Given that memory processing (acquisition, consolidation, storage, and retrieval) is a major function of the CNS, we determined if the 5hmC mark is differentially distributed in brain subareas involved in cognitive functions. We used a highly sensitive high-performance liquid chromatography/mass spectrometry (HPLC/MS) technique to answer this question (Fig. 2A). We measured the percentage 5hmC, 5mC, and 5C (unmodified cytosine) relative to total cytosine (5hmC + 5mC + 5C) in 5 different brain areas, and our measurements were consistent with earlier reported results (Globisch et al., 2010; Munzel et al., 2010). We found that 5hmC levels in area CA1 (0.70%) and cortex (0.74%) were significantly higher (*P* value < .0001) than in the dentate guyrus (0.63%), area CA3 (0.55%), and cerebellum (0.40%) (Fig. 2B). Cerebellum was found to have the lowest amount of 5hmC (0.40%) among the brain regions tested (Fig. 2B). In contrast to 5hmC, we found a fairly uniform distribution of the 5mC mark, ranging from 7% to 8% (Fig. 2C), and the percentage of unmodified cytosines, around 92%–93%, was also similar in the different brain areas (Fig. 2D). These results, consistent with other recent studies (Khare et al., 2012; Lister et al., 2013; Li et al., 2014), suggest the involvement of the 5hmC mark in learning and memory.

3.3. Tet1 deletion leads to a reduction in 5hmC levels

Tet proteins have been shown to convert 5mC to 5hmC (Tahiliani et al., 2009); therefore, we wanted to investigate if Tet1 contributes to establishing baseline 5hmC levels in the brain and if 5mC and 5C (cytosine) levels are also affected by *Tet1* ablation. To determine this, we subdissected WT and Tet1KO mice brain regions (CA1, DG, CA3, cortex, and cerebellum) and then measured 5mC, 5hmC, and 5C levels using a quantitative HPLC/MS technique. We found a significant reduction ($^{**}P < .05$, $^{**}P < .005$, and $^{****}P < .0001$) in 5hmC levels in Tet1KO mice in all brain areas investigated (Fig. 3A–E, left column). These data strongly support the idea that Tet1 acts catalytically on 5mC in the CNS to generate 5hmC (Kaas et al., 2013; Rudenko et al., 2013).

Because 5hmC can only be derived from oxidation of 5mC, it was possible that, in the absence of Tet1-mediated demethylation in Tet1KO mice, there might be an increase in relative 5mC levels given that we observed a decrease in 5hmC levels. However, we did not see a significant increase ($P > .05$) in 5mC levels except in the cortex ($^{*}P < .05$) (Fig. 3A–E, center column). Interestingly, in the cerebellum, both 5mC and 5hmC levels dropped significantly in Tet1KO mice ($^{*}P$ and $^{**}P < .05$) (Fig. 3E, left and center). We also did not observe a significant change ($P > .05$) in unmodified cytosine (5C) except in the case of cerebellum, where its levels in Tet1KO were slightly but significantly higher than those in the WT ($^{*}P < .05$) (Fig. 3A–E, right column). These results are consistent with a recently published study (Li et al., 2014) indicating that Tet1 may not be the sole regulator of active 5mC demethylation in the brain.

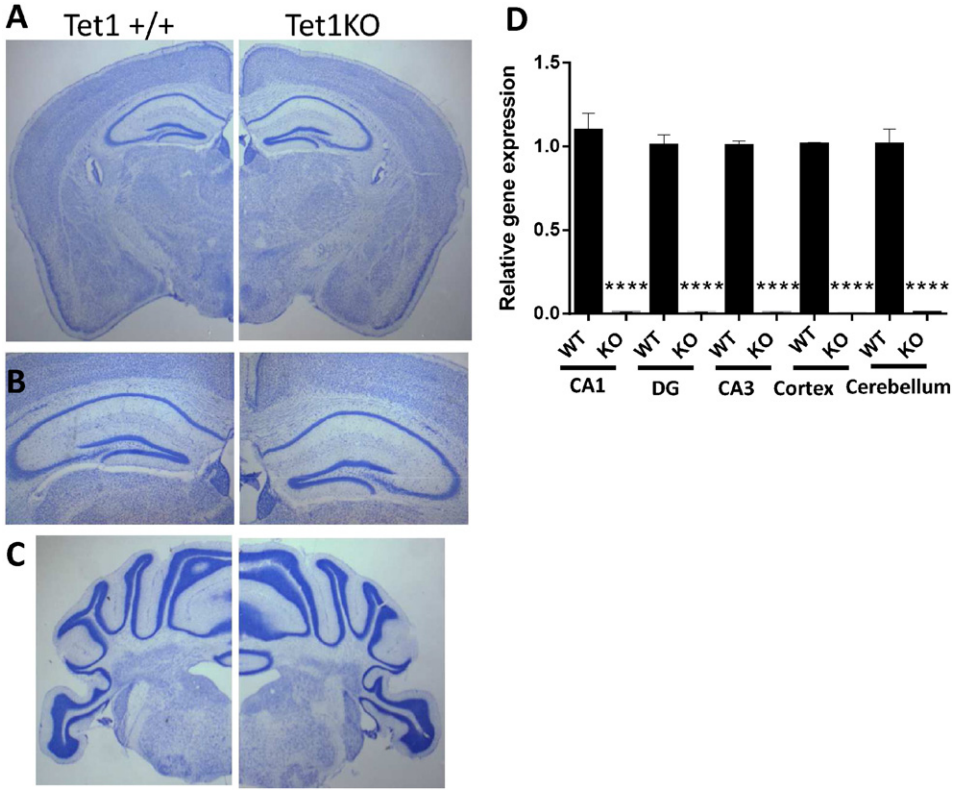


Fig. 1. Tet1KO mice have normal brain morphology (A–C). Cresyl violet staining of the coronal sections (50 μm) of Tet1 +/+ and Tet1KO mice brain showing cerebrum (A), hippocampus (B), and cerebellum (C). (D) Absence of Tet1 expression in the different brain areas of Tet1KO mice was confirmed by qRT-PCR using WT and Tet1KO animals; bars represent the Tet1 mRNA levels relative to WT (****$P < .0001$, n = 6–8 males per group); statistical comparisons were performed using an unpaired *t* test (2 tailed).

3.4. Tet1KO mice show normal baseline behaviors, motor memory, and social interactions

We performed a series of baseline behavior tests on Tet1KO mice to determine if *Tet1* deletion affects basic exploratory, emotional, and social behavior. In the open field paradigm, which measures general locomotor activity and anxiety, WT and Tet1KO did not show any significant differences ($P > .05$) in the total distance traveled and mean velocity (Fig. 4A). Also, both genotypes spent similar amounts of time in the center and along the walls of the chamber (Fig. 4B), suggesting a lack of difference in anxiety levels ($P > .05$). Another paradigm, the elevated plus maze, was also used to assess anxiety and depression-like behaviors. Tet1KO and WT did not show any significant differences ($P > .05$) in time spent in the open or closed arms, or in the number of entries made into each arm ($P > .05$) (Fig. 4C). Overall, these data indicate normal baseline locomotor and anxiety-related behavior in Tet1KO mice.

We also used a social approach paradigm described in Silverman et al. (2010) and Ellegood et al. (2013) using WT and Tet1KO animals to determine if Tet1KO mice exhibit any autistic-like behaviors. WT and Tet1KO showed no significant differences ($P > .05$) in their social interaction preferences; both preferred to spend more time with a novel mouse versus a novel object or empty chamber (Fig. 4D). Based on these results, we conclude that Tet1KO mice have normal exploratory and social behavior, at least as assessed using these protocols.

In terms of molecular changes, *Tet1* ablation elicited a significant decrease not only in 5hmC but also in 5mC levels in the cerebellum. We wanted to check if this reduction in the levels of both these cytosine derivatives was associated with any effect on cerebellum-dependent motor memory in Tet1KO mice. Using the accelerating rotarod paradigm, we found that Tet1KO and WT mice showed no significant differences in terms of the time to fall or velocity at fall in their rotarod performance on all 3 trial days (Fig. 4E). These observations suggest that Tet1KO mice have normal cerebellum-dependent motor memory.

3.5. Tet1KO mice exhibit enhanced threat recognition memory (contextual and cued fear conditioning)

Enrichment of 5hmC in brain areas (CA1 and Cortex) that are involved in memory processing propelled us to investigate Pavlovian threat recognition learning in Tet1-deficient animals. Therefore, we assessed both cued and contextual fear conditioning in Tet1KO animals versus littermate WT controls.

To evaluate long-term memory consolidation and storage, both WT and Tet1KO cohorts were trained with 2 different shock protocols, using "light" (0.5 mA for 1 second) or "strong" (0.8 mA for 2 seconds) training in a novel context. Memory retention was tested 24 hours after training. Tet1KO mice exhibited significantly higher freezing (*$P < .05$ and **$P < .005$) than littermate WT mice (Fig. 5B–C, left column) with both "light" and "strong" training paradigms. However, we observed no significant difference ($P > .05$) in freezing levels at 24 hours after robust training using a more intense shock protocol (0.8 mA, 2 seconds, repeated 3 times) (Fig. 5D), demonstrating that WT animals and Tet1KO animals have similar maximal learning capacities.

It has been shown that, 2 weeks after memory acquisition, memories undergo an extensive systems consolidation and are transferred to the cortex for long-term storage. These memories then become independent of the hippocampus and are referred to as *remote* memories (Dudai, 2004). To study if *Tet1* ablation affects remote memory systems consolidation and long-term storage of remote memories, WT and Tet1KO mice were tested in the training context 15 and 30 days after

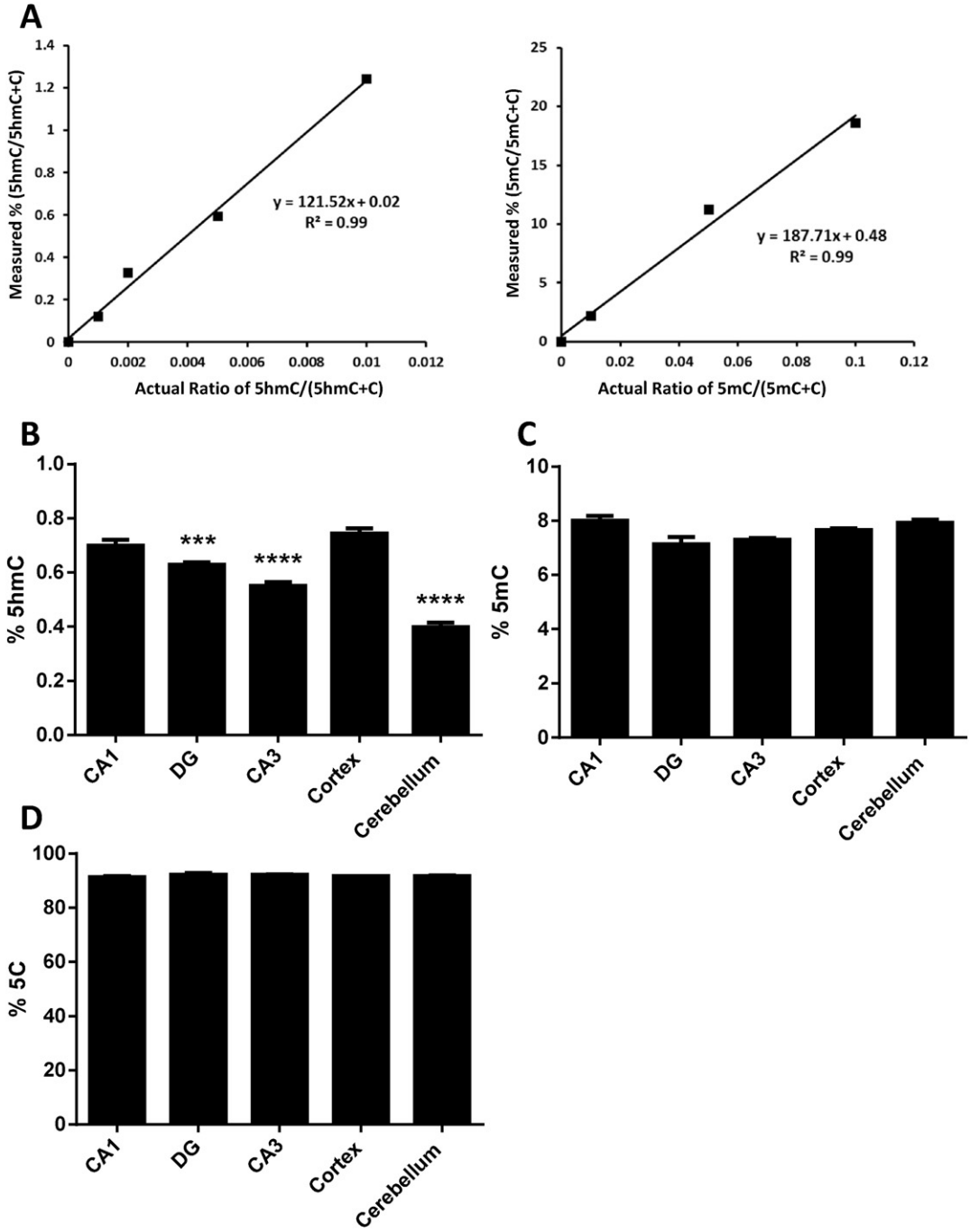
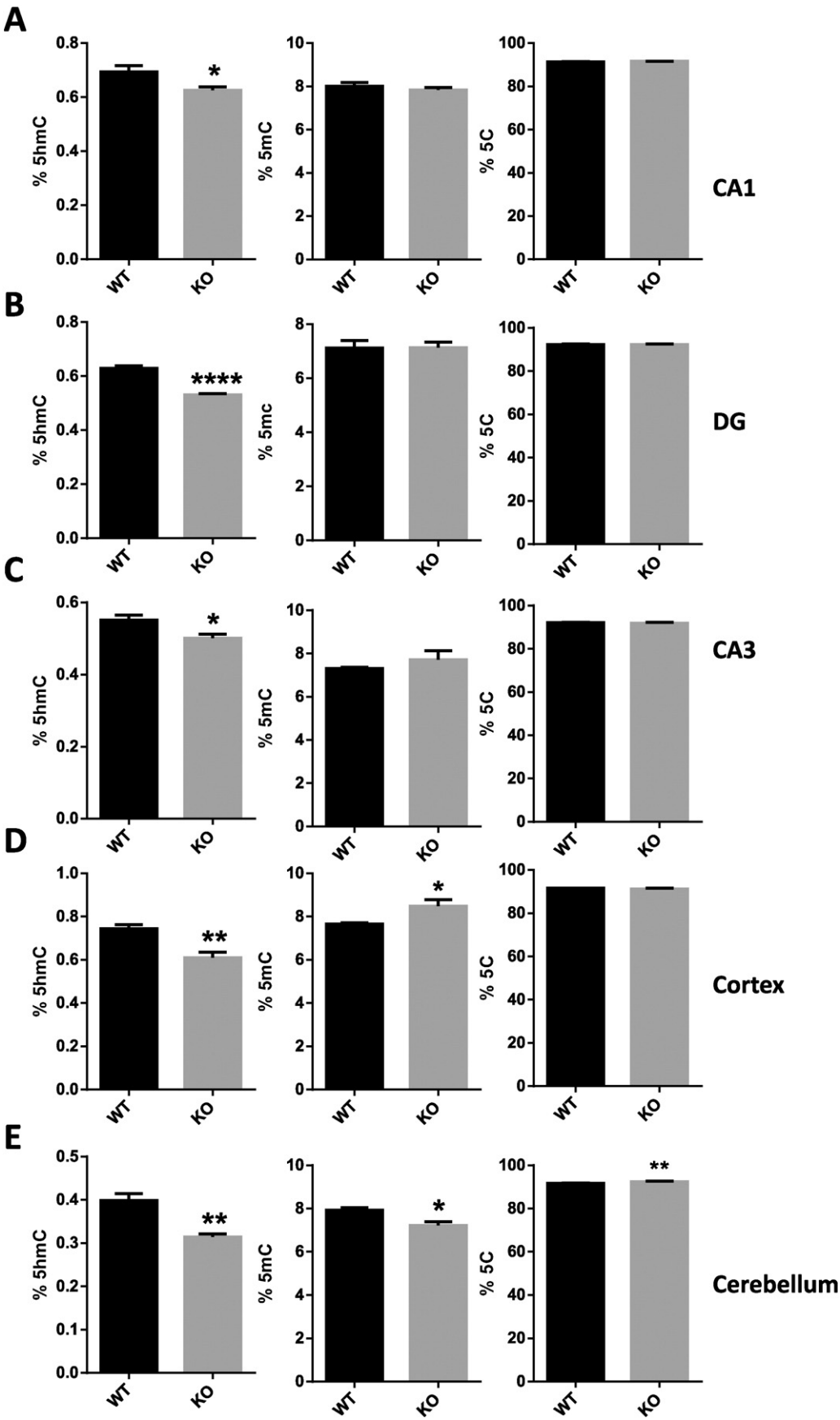


Fig. 2. 5hmC levels are enriched in brain areas involved in active memory formation and storage, whereas 5mC levels are more or less uniformly distributed in different brain regions. The quantification of the modified and unmodified cytosine bases was done by using highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) techniques. (A) Standard curves for 5mC and 5hmC. The percentages of 5mC and 5hmC are plotted against the known ratios of methylated and hydroxymethylated DNA to the total amount of cytosine in the standard samples. (B) Percentage 5hmC relative to total cytosine in different brain areas; cortex (0.74%) and CA1 (0.70 %) have significantly higher 5hmC levels (P value < .0001) than DG (0.63%), CA3 (0.55%), and cerebellum (0.40%). (C) No significant difference (P > .05) was observed in the percentage 5mC relative to total cytosine in different brain regions. (D) Percentage unmodified cytosine (5C) of the total cytosine in different brain regions (n = 4 males, error bars ± SEM). Statistical analysis was accomplished using one-way ANOVA with Bonferroni post hoc test.

"light" or "strong" training. Interestingly, in both cases, after 15 days (***P < .005, **P < .005) and after 30 days (***P < .0005, **P < .005), Tet1KO animals still showed significantly higher freezing levels than WT (Fig. 5B–C, center and right columns). Together, these results indicate an enhanced long-term remote memory consolidation and storage in Tet1KO mice.

Fig. 3. Tet1 loss leads to a reduction in 5hmC levels. Representative graphs of percentage 5hmC, 5mC, and 5C levels in the areas CA1, DG, CA3, cortex, and cerebellum determined using LC-MS/MS-MRM. (A–E) (left column) The levels of 5hmc in Tet1KO CA1, DG, CA3, cortex, and cerebellum are significantly lower than the WT CA1, DG, CA3, cortex, and cerebellum levels, respectively (*P < .05, **P < .005, ****P < .0001). (A–E) (center column) 5mC levels are significantly higher in Tet1KO cortex (*P < .05) and significantly lower in Tet1KO cerebellum (*P < .05), and no significant differences were found between 5mC levels of the areas CA1, DG, and CA3 of the Tet1 KO and WT mice. (A–E) (right column) 5C levels are slightly but significantly higher in Tet1KO cerebellum (**P < .05), and no significant differences were found between 5C levels of the areas CA1, DG, CA3, and cortex of the Tet1 KO versus WT mice. (n = 4 males per group, error bars ± SEM). Statistical comparisons were performed using an unpaired t test (2 tailed).


A
% 5hmC
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CA3
D
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% 5mC
% 5C
Cortex
E
% 5hmC
% 5mC
% 5C
Cerebellum
WT
KO

We also checked memory acquisition and short-term memory formation in Tet1KO animals. For this, we tested animals in the same context, 1 hour after the training. WT and Tet1KO did not show any significant difference ($P > .05$) in the percentage freezing 1 hour after training, indicating that fear learning and short-term memory are normal in Tet1KO mice (Fig. 5E).

We also investigated if *Tet1* ablation had an effect on hippocampus-independent cue memory in which animals learn to associate an auditory cue with a foot shock. For this paradigm, we trained WT and KO mice using the same "light" and "strong" foot-shock protocols that were used for the contextual training, however using an auditory cue (75 dB) as the conditioned stimulus. For testing, animals were exposed to a novel context for 5 minutes, 24 hours after training, during which the conditioned stimulus audio cue was played for the last 3 minutes (Fig. 6A). We observed significantly higher freezing ($^*P < .05$) in Tet1KO animals compared with WT mice using both "light" (Fig. 6B) and "strong" (Fig. 6C) protocols. We did not observe a significant difference ($P > .05$) in freezing with the "robust" foot-shock training protocol used as an additional control (Fig. 6D). Overall, these results show an enhancement in both hippocampus-dependent and -independent threat memory in Tet1KO mice.

3.6. Virally mediated knockdown of Tet1 expression in the hippocampus enhances spatial memory for object location

The use of fear conditioning training in a global knockout mouse line as described above does not allow for the determination of whether or not the role of TET1 oxidase in threat memory is limited to the hippocampus or stems from a synergistic effect of TET1 activity spanning several brain regions. Moreover, the enhanced memory phenotype in the Tet1KO mice may be a manifestation of potential developmental changes. For these reasons and to investigate whether observed phenotype is due to changes in neuronal function, an additional series of experiments was carried out, which combined the known hippocampus-selective, OLM task together with the acute reduction of *Tet1* expression in the dorsal hippocampus. We selectively targeted dorsal hippocampus because this region has been shown to be actively involved in spatial memory processing. Knockdown of *Tet1* mRNA levels was accomplished using AAVs engineered to express shRNAs specifically designed to target endogenous *Tet1* transcripts, as described in Guo et al. (2011).

To validate the effectiveness of our approach, we first stereotaxically injected either AAV-*Tet1*-shRNA viral particles or a scrambled control (scr-shRNA) into the dorsal hippocampus. At 2 weeks postsurgery, we observed robust expression throughout the dorsal CA1 and CA3 regions of the hippocampus (Fig. 7A). In addition, qRT-PCR analysis revealed a 46% reduction in *Tet1* mRNA levels in hippocampal tissue derived from AAV-*Tet1*-shRNA–injected mice compared with scr-shRNA controls ($^*P < .05$) (Fig. 7B). As a biochemical control for *Tet1* knockdown, we also measured the percentage of 5hmC and 5mC in microdissected hippocampal tissue from both groups using MS as described earlier. We did not see a significant decrease ($P > .05$) in the 5hmC levels in *Tet1*-shRNA infused mice compared with the control. However, consistent with a reduction in TET1 activity, we observed a trend ($P = .06$) towards a decrease in global 5hmC levels (Fig. 7C) in *Tet1*-shRNA–infused mice. No differences were found with regard to global 5mC levels (Fig. 7D).

Using the open field paradigm, we first tested whether a reduction in dorsal hippocampal *Tet1* expression in mice might affect their baseline behaviors or locomotion. We found no significant differences ($P > .05$) between the 2 groups in terms of total distance traveled or in time spent in the center or periphery of the open field. These results, consistent with our previous results with the Tet1KO mice, indicate that loss of *Tet1* expression does not affect basal exploratory and locomotor behavior in mice (Fig. 7E and F).

We next determined if *Tet1* knockdown in the dorsal hippocampus had any influence on OLM. For this task, *Tet1*-shRNA– and control scr-shRNA–infused mice were first exposed to 2 novel objects for a total of 10 minutes (Fig. 7G). Mice were then tested for long-term memory of the original location of the objects 24 hours later (Fig. 7I). Importantly, during training, a comparison of the percentage of time spent exploring each of the 2 objects did not differ significantly between groups (Fig. 7H), indicating no bias towards either object or its location in the chamber. In contrast, during the testing phase, mice receiving injections of AAV--*Tet1*-shRNA showed a statistically significant enhancement in memory for the object in the familiar location, and spent significantly more time ($^*P < .05$) exploring the object in the novel location, compared with AAV-scr-shRNA controls (Fig. 7J). These results are consistent with the idea that Tet1 may serve as a critical memory suppressor and that inhibition of Tet1 leads to persistence of hippocampus-dependent long-term memories.

3.7. Tet1KO mice have normal basal synaptic transmission and LTP, and virally mediated TET1 overexpression in dorsal hippocampus does not affect LTP

We next determined if enhanced long-term threat recognition memory is accompanied by a facilitation of LTP in Tet1KO mice. A recent study (Rudenko et al., 2013) showed normal LTP in Tet1KO mice using a relatively strong LTP induction protocol (2 episodes of theta burst stimulation, 10 bursts at 5 Hz, each with 4 stimuli at 100 Hz with 10-second intertrain intervals). To complement these prior studies, we used a relatively mild LTP induction stimulus (100 Hz, 1 second, delivered once) to check for the possibility of enhanced LTP in Tet1KO mice at near-threshold LTP induction stimuli. However, we did not observe any significant enhancement ($P > .05$) in LTP measured in area CA1 using this stimulus (Fig. 8A, bottom). Neither did we observe enhanced LTP using a subthreshold stimulus intensity under our conditions (Fig. 8B, bottom). We also did not find any significant difference in PPF ($P > .05$), suggesting that both WT and Tet1KO have normal presynaptic release probability (Fig. 8A–B, middle). Basal synaptic transmission was also found to be normal in Tet1KO mice (Fig. 8A–B, top).

To further investigate if Tet1 plays a role in regulating LTP, we used the virus-mediated overexpression approach described in Guo et al. (2011) and Kaas et al. (2013). Thus, we stereotaxically injected AAVs overexpressing a hemagglutinin-tagged catalytic domain of human TET1, or a catalytically inactive version (TET1m), into the dorsal hippocampus. At 2 weeks postinfection, hippocampal slices were prepared for LTP assessment. Long-term potentiation was induced using 1-time theta burst stimulation (1 episode of theta burst stimulation, 10 bursts at 5 Hz, each with 4 stimuli at 100 Hz with 20-second intervals). We found that the overexpression of either the catalytically active (AAV-TET1) or the catalytically inactive (AAV- TET1m) TET1 peptide did not lead to any significant effect on LTP compared with control AAV-eYFP (Fig. 8C, bottom) ($P > .05$). In addition, there were no significant differences in basal synaptic transmission ($P > .05$) (Fig. 8C, top) and PPF (Fig. 8C, middle) between the AAV-TET1–, AAV-TET1m, and AAV-eYFP–infused mice.

Overall, these results indicate that despite Tet1KO mice displaying enhanced behavioral memory, hippocampal LTP remains normal in these animals—replicating the prior results of Rudenko et al. (2013) using 2 additional LTP induction protocols and in addition testing an independent assessment of the effects of TET1 overexpression. Taken together, these various observations strongly suggest that the behavioral effects of Tet1 knockout or overexpression do not involve hippocampal LTP but rather involve some other form of synaptic or cellular plasticity.

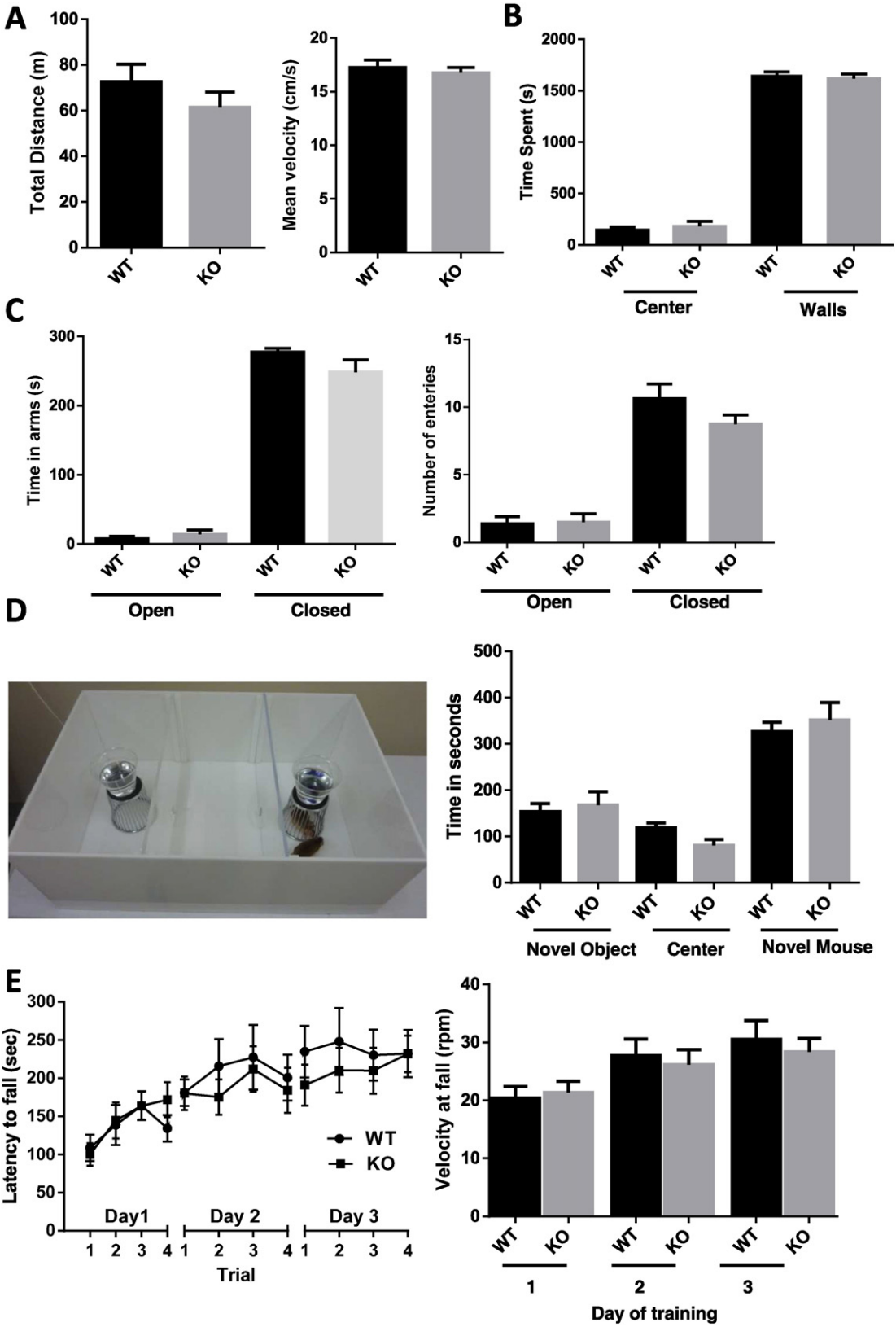

Fig. 4. Tet1 KO mice show normal baseline and social behavior. (A) Open field. No significant differences were found in the horizontal activity measured as distance travelled (left) and velocity (right) between WT and Tet1KO. (B) No significant differences were found between WT and Tet1KO in terms of time spent in the center versus the time spent in periphery taken as a measure of anxiety. (C) Elevated plus maze. No significant differences were found between WT and Tet1KO in the time spent in the open and closed arms and the number of entries made to each arm of the maze. (D) Both WT and Tet1KO exhibited normal social interaction and preferred spending time with novel mouse over novel object and central chamber. (E) No significant differences were found between the performance of the WT and Tet1KO in the accelerating rotarod test; left column shows the time to fall, and right column represents the velocity at fall (n = 8 males per group, error bars ± SEM). Statistical comparisons between 2 groups were performed using an unpaired *t* test (2 tailed). Statistical analysis between 3 or more groups was accomplished using 1-way ANOVA with Bonferroni post hoc test.

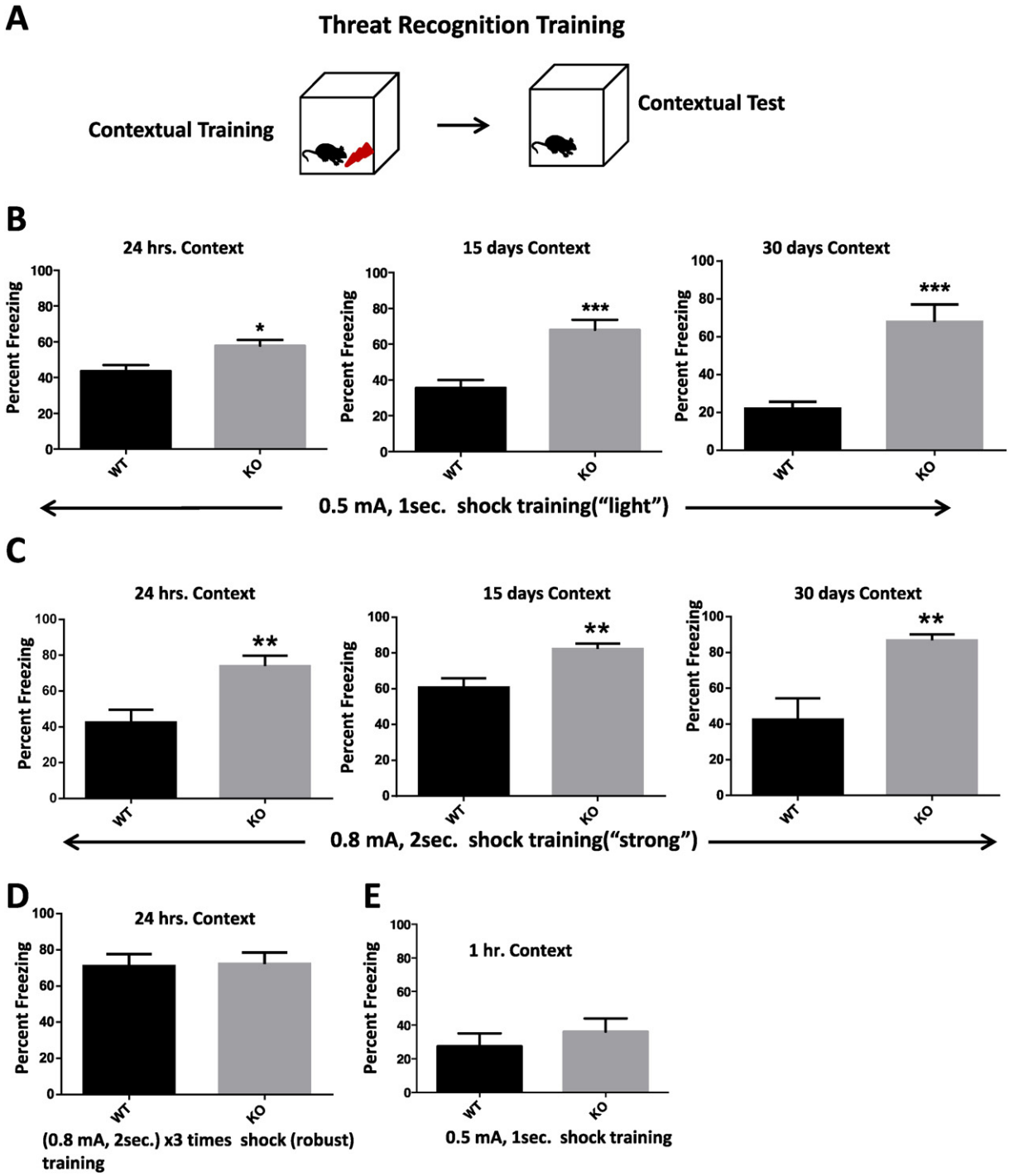

Fig. 5. Tet1KO has enhanced long-term memory for contexual threat recognition training (fear conditioning). (A) Schematic diagram of the contextual threat recognition training. (B) Tet1 KO showed significantly higher freezing than WT in context testing after 24 hours (*$P < .05$), 15 days (***$P < .005$), and 30 days (***$P < .0005$) of "light" training (0.5 mA for 1 second). (C) Tet1 KO showed significantly higher freezing than WT in context testing after 24 hours (**$P < .005$), 15 days (**$P < .005$), and 30 days (**$P < .005$) of "strong" training (0.8 mA for 2 seconds). (D–E) No significant difference in freezing was observed between WT and Tet1KO in context testing after 24 hours of robust training (0.8 mA, 2 seconds, 3 times) and 1 hour of "light" training (0.5 mA for 1 second) (n = 8 males per group, error bars ± SEM). Statistical comparisons were performed using an unpaired *t* test (2 tailed).

3.8. Tet1 deletion leads to altered transcription of memory-associated genes

De novo transcription and translation of activity-regulated genes are required for long-term memory storage. A variety of neuronal genes regulate neural plasticity in response to a learning experience, and altered transcription of these genes is crucial in neural development, learning, and memory (Leslie and Nedivi, 2011; West and Greenberg, 2011). We used qRT-PCR to investigate the expression of several activity-induced genes that have been implicated in various forms of synaptic plasticity and synapse development in the different brain areas of Tet1KO mice under study: CA1, CA3, DG, cerebral cortex, and cerebellum.

We observed a significant decrease (*$P < .05$, ****$P < .0001$) in the expression of *Arc* (activity-regulated cytoskeleton-associated protein) in all brain areas except cerebellum (Fig. 9). Interestingly, *Egr1* (early growth response protein 1) was significantly decreased in CA1 (***$P < .0005$),

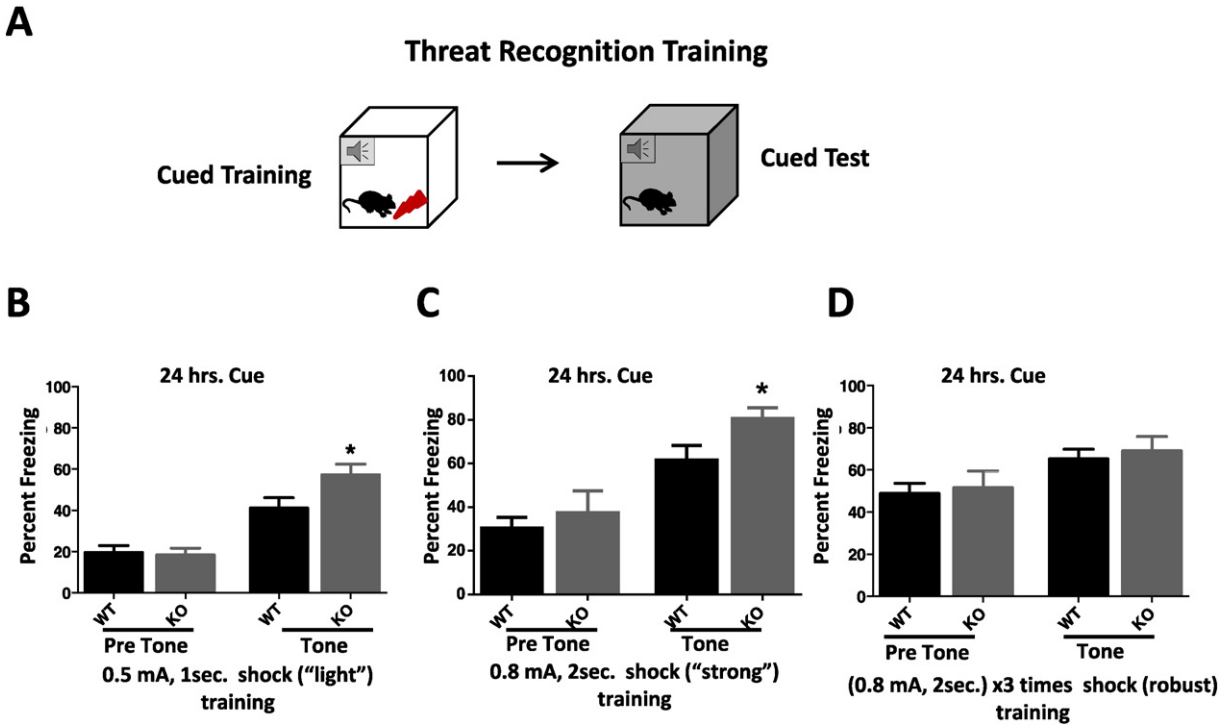

Fig. 6. Tet1KO has enhanced long-term memory for cue threat recognition training (fear conditioning). (A) Schematic diagram of cue threat recognition training. (B–C) Tet1KO showed significantly higher freezing (*$P < .05$) in the cue testing after 24 hours of cue training using "light" (0.5 mA for 1 second) and "strong" training (0.8 mA for 2 seconds) protocol. (D) No significant difference ($P > .05$) in freezing was observed between WT and Tet1KO in cue testing after 24 hours of "robust" training (0.8 mA, 2 seconds, 3 times). (n = 8 males per group, error bars ± SEM). Statistical comparisons were performed using an unpaired *t* test (2 tailed).

but was significantly increased in cerebellum (**$P < .005$), and did not show any significant change in expression in other brain areas (Fig. 9). A significant increase (*$P < .05$, **$P < .005$) in the expression of *Creb1* (cAMP responsive element binding protein 1) was observed in CA1, cortex, and cerebellum (Fig. 9). We also probed *Bdnf* (brain-derived neurotrophic factor) transcripts by using exon IX primers (an exon present in all the expressed isoforms) and found a significant increase (***$P < .0005$) in the expression of *Bdnf* in all the brain areas examined except cortex (Fig. 9). *c-Fos* (FBJ osteosarcoma oncogene) was significantly downregulated in area CA1, DG, and cortex. A significant increase in *calcineurin* expression (*$P < .05$, **$P < .005$) was seen in all the brain areas examined (Fig. 9). *reelin* (*reln.*) was significantly upregulated (*$P < .05$, **$P < .005$) only in DG and cerebellum (Fig. 9). Interestingly, *Homer1* (homer protein homolog 1) showed a significant downregulation (*$P < .05$) in area CA1 and cortex, and a significant upregulation (*$P < .05$) in area CA3 (Fig. 9). *Cdk5* (cyclin-dependent kinase 5) was significantly upregulated (*$P < .05$, **$P < .005$) in all brain areas except DG (Fig. 9). *Nr4a2* (nuclear receptor related 1 protein) was also significantly upregulated (*$P < .05$, **$P < .005$, and ****$P < .0001$) in all brain areas except cerebellum (Fig. 9). *NPas4* (neuronal PAS domain protein 4) was significantly downregulated (**$P < .005$, ***$P < .0005$, ****$P < .0001$) in all the brain areas examined except CA3 (Fig. 9). These results show that genetic deletion of *Tet1* leads to an extensive alteration in the expression of crucial neuronal-activity regulated genes, in a wide variety of memory-associated brain regions, and that Tet1 may positively or negatively regulate the same gene depending upon the brain area.

3.9. Tet1 regulates the transcription of Tet2, Tet3, and other active demethylation pathway genes

Tet1 clearly has a role in maintaining 5hmC levels in the brain, as illustrated by our observation that loss of Tet1 led to a significant reduction in the levels of 5hmC in memory-associated brain subregions (Fig. 3). However, the loss of Tet1 and the reduction in the level of 5hmC do not translate to a significant increase in 5mC in Tet1KO mice, except in the cortex (Fig. 3). This suggests the possibility that Tet2 and Tet3 might be playing a compensatory role in the absence of Tet1, restoring normal baseline cytosine methylation levels, even in the face of loss of one driver of active demethylation. Therefore, we checked the expression of *Tet2* and *Tet3* mRNAs in different brain areas of Tet1KO mice by qRT-PCR and found a significant (*$P < .05$, **$P < .005$, ***$P < .0005$, ****$P < .0001$) compensatory increase in the expression of both the transcripts (Fig. 10).

As described in the introduction, mounting evidence now points to the existence of an active demethylation pathway in cells (Fig. 10A), and the components of this pathway are conserved in the brain. The active demethylation pathway cycle consists of cytosine methylation by DNA methyltransferases (DNMTs), repetitive oxidation of the methyl group by Tet proteins (TET1, 2, and 3), and restoration of unmodified cytosines by DNA glycosylase mediated base excision repair or by nucleotide excision repair (Wu and Zhang, 2014) (Fig. 10A). We decided to check if there is a compensatory alteration in the expression of the various genes involved in this pathway in various regions of the Tet1KO brain. We found a significant increase (*$P < .05$, **$P < .005$, ***$P < .0005$, ****$P < .0001$) in the expression of several genes involved in this pathway (Fig. 10): *Gadd45b* (growth arrest and DNA-damage-inducible beta), *Smug1* (single-strand selective monofunctional uracil DNA glycosylase), *Apobec1* (apolipoprotein B mRNA-editing enzyme 1), and *Tdg1* (thymine DNA glycosylase 1) were all upregulated in all the different brain areas investigated (Fig. 10). Interestingly, *Gadd45b* has also been characterized as a neuronal activity–induced immediate early gene (Ma et al., 2009). From these results, we conclude that Tet1KO mice have an overall compensatory upregulation of various active demethylation genes including *Tet2* and *Tet3*, suggesting a pathway in cells allowing the coordinated genetic regulation of the entire machinery of active DNA demethylation.

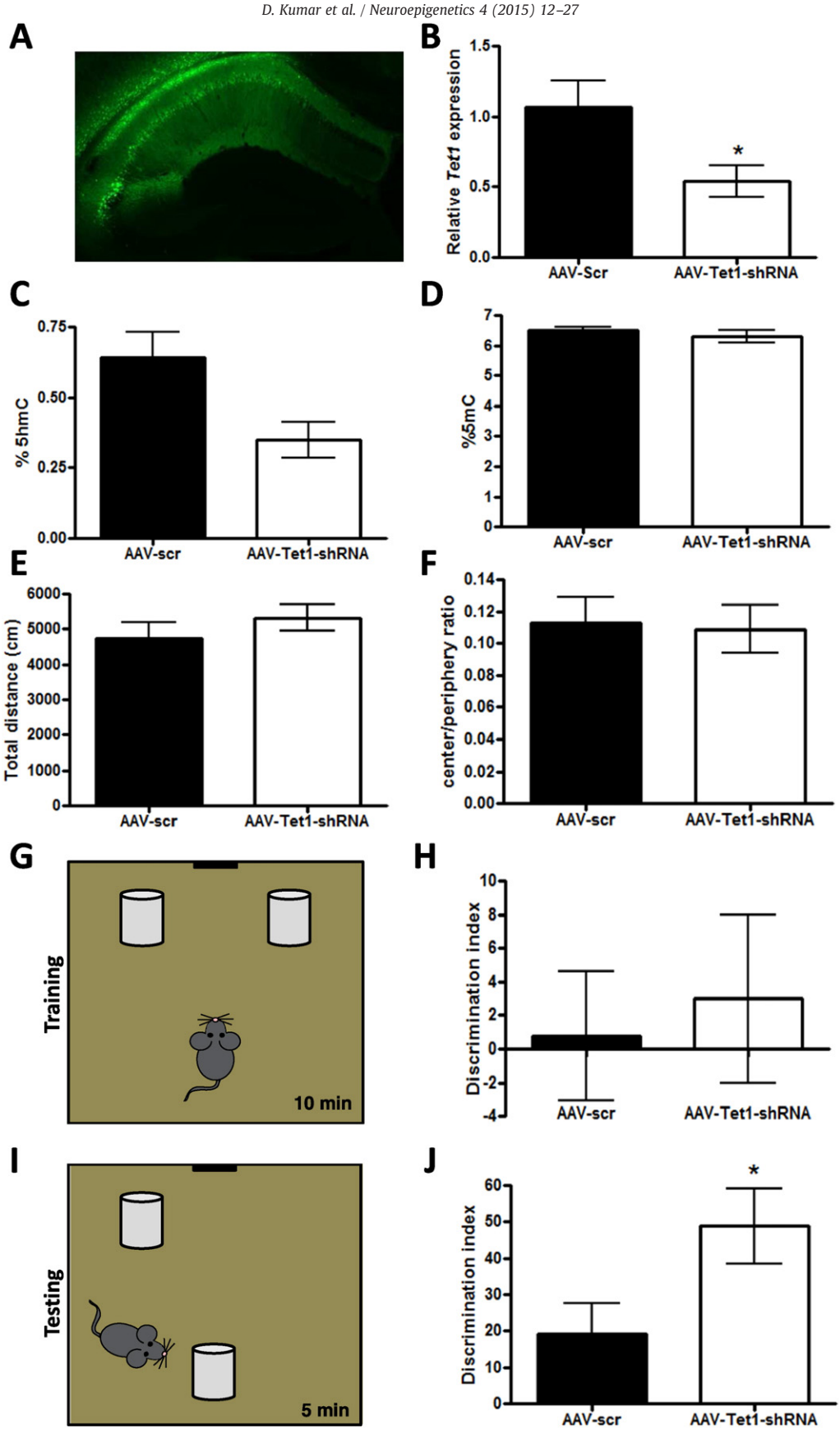

Fig. 7. Virally mediated reduction in hippocampal Tet1 mRNA enhances spatial memory. (A) A representative image of AAV-mediated shRNA transgene expression; 14 days postinjection. (B) Quantitative RT-PCR analysis comparing the levels of Tet1 mRNA in mice 14 days following injection with either AAV-Tet1-shRNA or AAV-scrambled-shRNA control (n = 3–5 per group). (C) Global 5hmC and (D) global 5mC percentages relative to total cytosine content, as measured by HPLC/MS (n = 3 males per group). (E) Total distance traveled during 15 minutes in the open field. (F) The ratio of time spent in the center to time spent in the periphery of the open field, an indirect measure of anxiety. (G) (left) A schematic diagram of OLM training. (H) Discrimination index for each group during training. (I) A schematic diagram of the OLM test. (J) Discrimination index 24 hours after OLM training. For figure panels E–J, n = 8 for each group. Where applicable, $^{*}P < .05$, unpaired *t* test (two tailed). All data are presented as ± SEM.

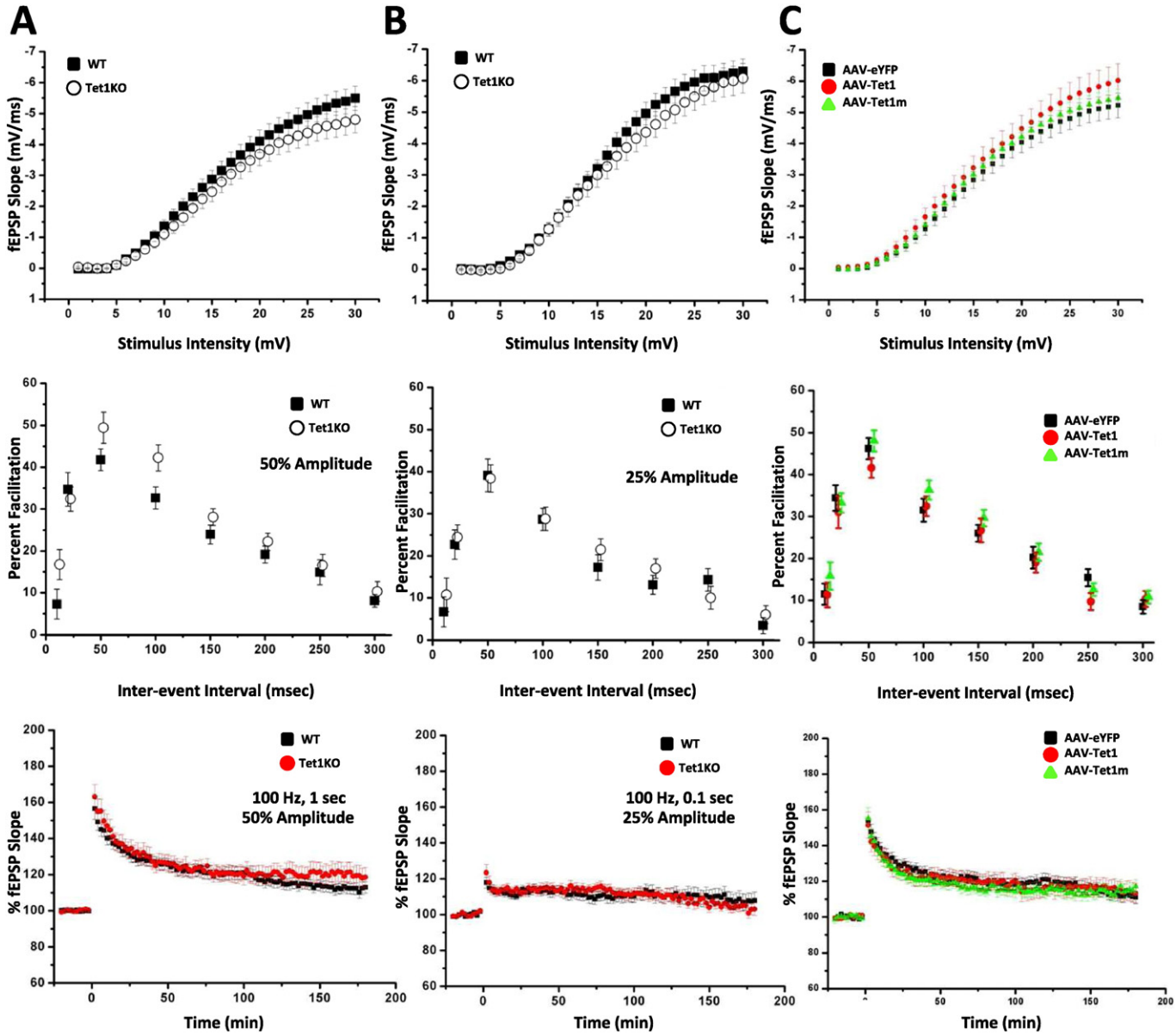

Fig. 8. Hippocampal LTP in Tet1KO mice and in mice with virally mediated Tet1 overexpression. (A–B) (top) No significant differences were found in baseline synaptic transmission between WT and Tet1KO, as indicated in these plots representing input-output relationship of evoked fEPSP slope versus stimulus intensity. (C) (top) No significant differences were found in baseline synaptic transmission between AAV-Tet1, AAV-Tet1m, and AAV-eYFP overexpressing mouse hippocampal brain slices. (A–B) (middle) No significant differences were found in PPF using different interevent interval stimuli with normal and low-intensity stimulation. (C) (middle) No significant differences were found in PPF between AAV-Tet1, AAV-Tet1m, and AAV-eYFP overexpressing mouse hippocampal brain slices using normal amplitude stimulus intensity. (A–B) (bottom) No significant differences were found in LTP between WT and Tet1KO. fEPSPs were recorded from area CA1 before and after tetanic stimuli (100Hz, 1 second, 50% of the maximum slope and 100 Hz, 0.1 second, 25% of the maximum slope) of Schaffer collaterals. (C) (bottom) No significant differences were found in the LTP between AAV-Tet1, AAV-Tet1m, and AAV-eYFP overexpressing mice hippocampal brain slices using theta burst stimulation. (n = 6 males per group, 7 slices each, error bars ± SEM). Statistical comparisons between 2 groups were performed using an unpaired *t* test (2 tailed); statistical analysis between 3 or more groups was accomplished using 2-way ANOVA.

3.10. Tet1 deletion leads to upregulation of other epigenetic modifiers

Different epigenetic marks (5mC, 5hmC, 5fC, and 5caC) have been shown to interact with common and specific epigenetic reader proteins that have gene regulation capability (Iurlaro et al., 2013; Spruijt et al., 2013). For this reason, we analyzed the expression of different epigenetic modifiers after the ablation of *Tet1*, including DNMTs and methyl cytosine binding proteins. Interestingly, we found that Tet1KO mice manifest significant increases ($^{*}P < .05$, $^{**}P < .005$, $^{***}P < .0005$, and $^{****}P < .0001$) in the expressions of *Dnmt1*, *3a*, *3b* (DNA methyltransferases); *Mecp2* (methyl CpG binding protein 2); and *Mbd3* and *4* (methyl-CpG-binding domain protein) transcripts in the 5 brain regions under study (the only exception being no change in *Mbd4* transcripts in CA1 and CA3) (Fig. 11). This observation also suggests the existence of coordinated transcriptional regulation of genes contributing to the pathways subserving epigenomic regulation in the CNS.

4. Discussion

A growing body of evidence suggests that DNA methylation in the adult brain is dynamically regulated and crucial for controlling memory formation and storage (Sweatt, 2013). In this regard, our studies further establish Tet1 oxidase as a driver of active demethylation in the adult CNS and a controller of memory consolidation and stabilization.

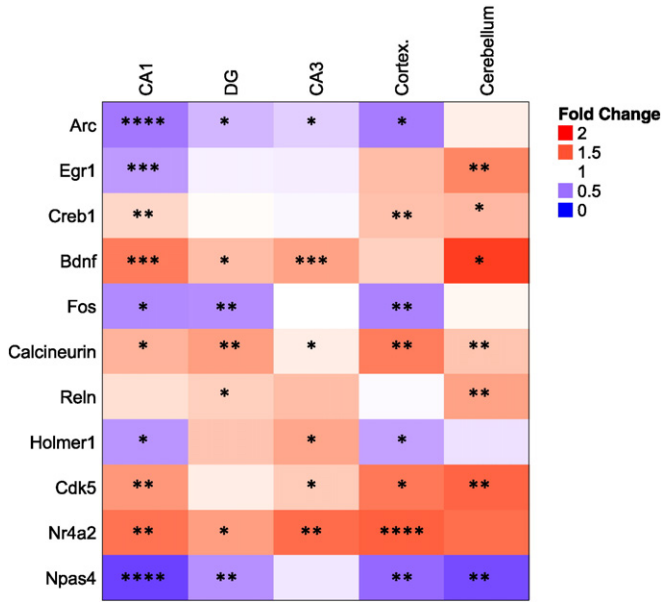

Fig. 9. Tet1KO has altered expression of neuronal activity-regulated genes. The heat map represents the qRT-PCR analysis of the mRNA transcripts. Color key represents relative gene expression (fold change) of the labeled gene in Tet1KO compared with the WT control. The "stars" represent the significance (*$P < .05$, **$P < .005$, ***$P < .0005$, ****$P < .0001$, n = 8 males per group, error bars represented as ± SEM). Statistical comparisons were performed using an unpaired *t* test (2 tailed).

In the present studies, we found the presence of 5hmC throughout the brain; however, we observed a significant enrichment of 5hmC levels in hippocampal area CA1 and cortex (Fig. 2B). Because CA1 and cortex are parts of the brain actively involved in memory processing, the specific enrichment of 5hmC in these areas is consistent with a role for 5hmC in cognitive function. Other recent studies using genome-wide sequencing have also pointed at the involvement of 5hmC in learning and memory (Khare et al., 2012; Lister et al., 2013; Li et al., 2014).

Tet1KO mice showed lower levels of 5hmC than WT in the different brain regions we assessed (Fig. 3A–E, left column), data that are directly supportive of the hypothesis that Tet1 catalyzes the formation of 5hmC (Tahiliani et al., 2009). However, deletion of TET1 did not lead to significant accumulation of 5mC except in cortex (Fig. 3A–E, center), suggesting possible compensation by TET2 and TET3 to restore normal levels of 5mC in DNA through ongoing demethylation. This interpretation is consistent with a recent study by Li et al. (2014) showing the involvement of TET3 in mediating rapid behavioral adaptation in the prefrontal cortex.

One striking discovery in the present studies is the observation of memory enhancement in Tet1KO animals, particularly regarding threat recognition, long-term memory, and remote memory consolidation. Consistent with a previous study (Rudenko et al., 2013), we found normal threat memory acquisition in Tet1KO mice and that short-term threat memory was also normal in Tet1KO mice (Fig. 5E). However, we found an enhancement in memory consolidation and long-term storage of hippocampus- and cortex-dependent memories in Tet1KO mice (Fig. 5B–C). We also found an enhancement in the hippocampus-independent, amygdala-dependent cue memory in Tet1KO mice (Fig. 6A–D). We further showed that a virus-mediated shRNA knockdown of *Tet1* in dorsal hippocampus led to an enhanced hippocampus-dependent long-term spatial memory for object location (Fig. 7J). Recently, Zhang et al. (2013) reported that a deletion of Tet1 resulted in a hippocampus-dependent spatial memory impairment as assessed by the Morris water maze task. These observations suggest a different role of Tet1 in different memory types. Although both Morris water maze and contextual fear conditioning are hippocampal-dependent tasks, Morris water maze may involve stronger and more aversive motivational factors than fear conditioning and occurs over many more training trials of longer duration. These differences might account for differential susceptibilities to effects of Tet1 knockout in the water maze versus fear conditioning. Overall, however, these new findings establish Tet1 as a regulator of associative conditioning and spatial memory.

The cellular mechanisms through which Tet1 oxidase and active demethylation regulate memory formation and consolidation remain mysterious. However, the present results along with others recently published (Kaas et al., 2013; Rudenko et al., 2013) make it clear that hippocampal LTP is not a strong candidate to subserve this function. Thus, in the present studies, *Tet1* gene deletion and TET1 catalytic domain overexpression in mice did not alter LTP (Fig. 8A–C). These findings suggest that TET1 is acting via some other form of synaptic or cellular plasticity. Two specific possibilities are: altered synaptic LTD (Rudenko et al., 2013) or altered neuron-wide synaptic homeostasis (Sweatt, 2013; Meadows et al., 2015; Yu et al., 2015). Future studies will hopefully yield further insights into this important issue.

In our studies of transcriptional regulation in Tet1KO mice (Fig. 9), we investigated the expression of a wide variety of known memory-associated genes in Tet1KO mice. A number of these genes (*Arc*, *Bdnf*, *calcineurin*, *reelin*, and *Npas4*) have previously been shown either to be epigenetically regulated or to interact with cellular epigenetic signaling (Miller and Sweatt, 2007; Lubin et al., 2008; Miller et al., 2010; Penner et al., 2011; Rudenko et al., 2013). Interestingly, in the present studies, we found that the deletion of *Tet1* can lead to downregulation or upregulation of the same memory-associated gene depending upon the brain area; for example, *Egr1* is downregulated in CA1 and upregulated in cerebellum, and *Homer1* is downregulated in CA1 and cortex, whereas it is upregulated in CA3. We found a significant increase in *Creb1* in CA1 and in cortex; Creb1 is an important component of the cAMP pathway and has been shown to be a positive regulator of long-term memory storage (Dash et al., 1990; Bartsch et al., 1998). We also found a significant increase in the expression of 2 important Creb1 targets, *Bdnf* (in CA1) and *Nr4a2* (in both CA1 and Cortex). Both *Bdnf* and *Nr4a2* expressions have been shown to increase following treatment with HDAC inhibitors that result in memory enhancement, and blocking Nr4a signaling affects the ability of HDAC inhibitors to enhance memory (Vecsey et al., 2007; Hawk et al., 2012). Finally, we observed a significant increase in *Cdk5* transcription in both CA1 and cortex, and recently, Cdk5 has been implicated in the cAMP pathway (Guan et al., 2011).

Our results investigating altered gene transcription in Tet1KO mice demonstrate broad secondary changes in the transcription of genes encoding epigenomic signaling enzymes including components of both the cytosine methylation and demethylation pathways. These findings suggest the model that active DNA demethylation pathways are coordinately regulated at the transcriptional level, perhaps as a cellular form of homeostatic plasticity at the genomic level. Consistent with this idea, we found that, in Tet1KO mice, not only *Tet2* and *Tet3* but also other known genes involved in the cytosine demethylation pathway (*Gadd45b*, *Smug1*, *Apobec1*, and *Tdg*) are significantly upregulated (Fig. 10). Interestingly, a recent study shows distinct roles of Tet1 and Tet2 in mouse embryonic stem cells, Tet1 mainly controlling 5hmC levels at gene promoters and transcription start sites, whereas Tet2 maintains 5hmC levels in gene bodies and at exon boundaries (Huang et al., 2014). In the future, it will be interesting to determine if different *Tet* isoforms similarly perform different roles in epigenomic structure in the CNS (Li et al., 2014). However, the current results and those of Kaas et al. (2013) strongly indicate the coordinated expression of genes encoding major components of the active DNA demethylation pathway.

Our results are in good agreement with recently published findings from the Tsai and Jaenisch laboratories characterizing the same line of *Tet1*-deficient mice. Specifically, our results independently replicate

their findings concerning normal brain morphology; altered 5-hmC production; normal baseline behaviors; normal hippocampal synaptic transmission and LTP; and altered *Arc*, c-*Fos*, and *Npas4* gene transcription in *Tet1*−/− mice. Thus, our work complements these previous findings and also extended them by investigating additional brain subregions, social interaction behavior, and motor learning, thereby revealing an important new attribute of the *Tet1*−/− mouse line, which is enhanced long-term and remote threat recognition memory (fear conditioning). Interestingly, while our paper was in revision, a similar study came out from Nestler Lab (Feng et al., 2015), however focusing on the role of Tet1 and 5hmC in cocaine action in the nucleus accumbens. They reported a downregulation of Tet1 in nucleus accumbens in response to long-term cocaine administration and also showed, using virally mediated manipulations, that Tet1 acts as a negative regulator of cocaine reward memory.

Considering transcription changes vis-à-vis the memory enhancement we observed, there are several specific possibilities that may have contributed to the enhanced memory consolidation and storage in Tet1KO. These include the (1) upregulation of Creb1 (a positive regulator of memory storage) and other activity-regulated genes involved in the cAMP pathway as described before; (2) decrease in Npas4 expression in Tet1KO mice, which might have led to an increase in the number of excitatory circuits resulting in the enhancement in memory consolidation and storage because Npas4 is a transcription factor that has been implicated in inhibitory synapse development (Lin et al., 2008; Bloodgood et al., 2013); and (3) increased expression of DNMTs in Tet1KO mice, which might have led to a secondary increase in memory capacity because DNMT inhibition has been shown to diminish memory consolidation and storage. These possibilities are not mutually exclusive, and all the 3 changes might have together altered the epigenetic state of the Tet1KO brain such that memory consolidation and storage are favored.

Overall, our results implicate Tet1 as a suppressor of memory formation. Because the Tet1KO mice are developmentally normal and have no obvious changes in baseline behavior or motor activity yet exhibit memory enhancement, Tet1 inhibition may serve as a strong therapeutic target for memory restoration in neurodegenerative disorders such as Alzheimer disease, in age-related cognitive decline, or in intellectual disability syndromes.

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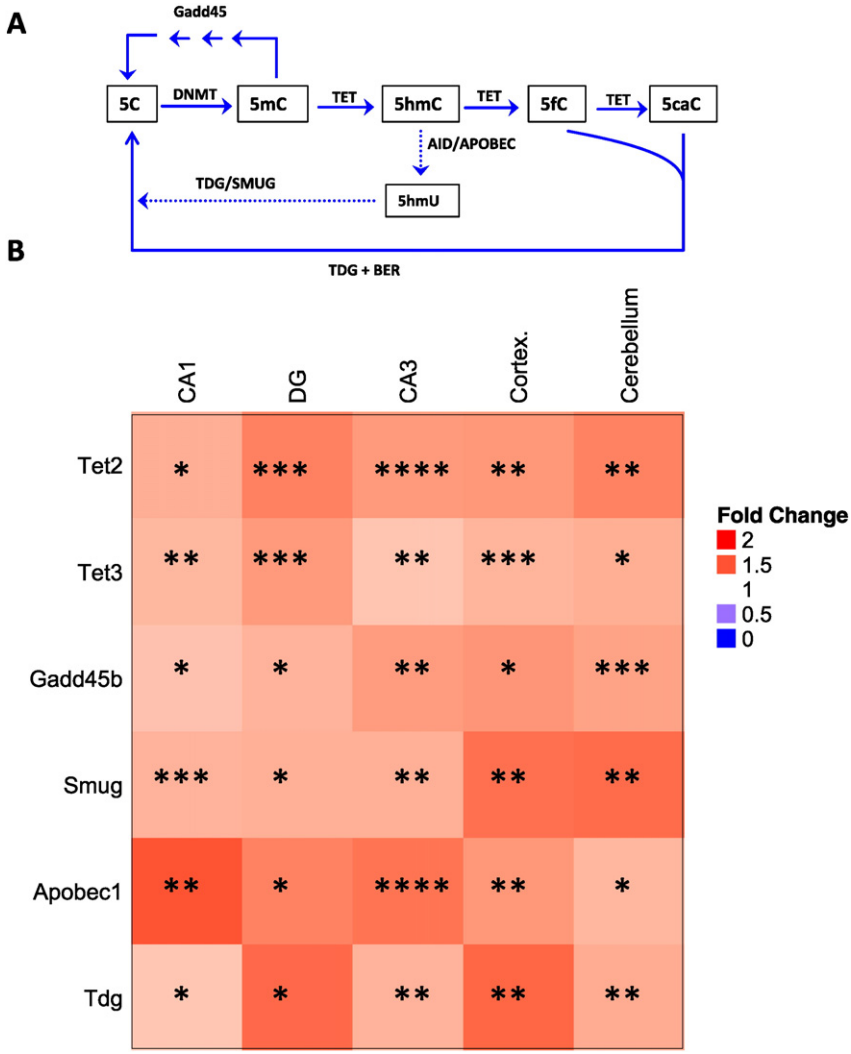

Fig. 10. Tet1KO has compensatory upregulation of various active demethylation pathway genes. (A) Schematic presentation of the active DNA demethylation pathway in the adult CNS. (B) The heat map represents the qRT-PCR analysis of the mRNA transcripts. Color key represents relative gene expression (fold change) of the labeled gene in Tet1KO compared with the WT control. The "stars" represent the significance (*$P < .05$, **$P < .005$, ***$P < .0005$, ****$P < .0001$, n = 8 males per group, error bars represented as ± SEM). Statistical comparisons were performed using an unpaired *t* test (2 tailed).

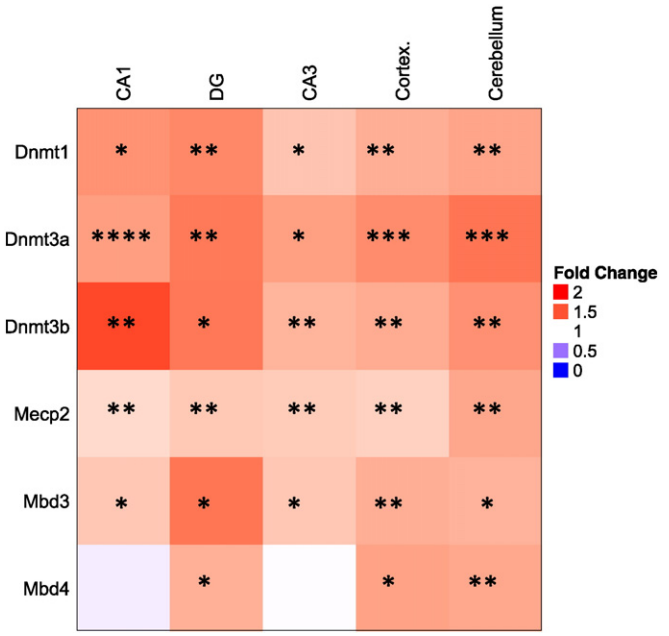

Fig. 11. Genetic deletion of Tet1 leads to an increase in the expression of various epigenetic modifiers. The heat map represents the qRT-PCR analysis of the mRNA transcripts. Color key represents relative gene expression (fold change) of the labeled gene in Tet1KO compared with the WT control. The "stars" represent the significance ($^{*}P < .05$, $^{**}P < .005$, $^{***}P < .0005$, $^{****}P < .0001$, n = 8 males per group, error bars represented as ± SEM). Statistical comparisons were performed using an unpaired *t* test (2 tailed).

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