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Tet1 Is Critical for Neuronal Activity-Regulated Gene Expression and Memory Extinction

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SUMMARY

The ten-eleven translocation (Tet) family of methylcytosine dioxygenases catalyze oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and promote DNA demethylation. Despite the abundance of 5hmC and Tet proteins in the brain, little is known about the functions of the neuronal Tet enzymes. Here, we analyzed Tet1 knockout mice (Tet1KO) and found downregulation of multiple neuronal activity-regulated genes, including *Npas4*, c-*Fos,* and *Arc*. Furthermore, Tet1KO animals exhibited abnormal hippocampal long-term depression and impaired memory extinction. Analysis of the key regulatory gene, *Npas4*, indicated that its promoter region, containing multiple CpG dinucleotides, is hypermethylated in both naive Tet1KO mice and after extinction training. Such hypermethylation may account for the diminished expression of *Npas4* itself and its downstream targets, impairing transcriptional programs underlying cognitive processes. In summary, we show that neuronal Tet1 regulates normal DNA methylation levels, expression of activity-regulated genes, synaptic plasticity, and memory extinction.

INTRODUCTION

DNA methylation is a covalent modification that is critical for the regulation of gene expression in a wide variety of biological contexts (Jaenisch and Bird, 2003; Bergman and Cedar, 2013). While methylation of DNA at 5-cytosine residues, as well as the methyltransferase (DNMT) enzymes that are responsible for this process, have been relatively well characterized (Jaenisch and Bird, 2003; Feng et al., 2010), the complementary process of DNA demethylation remains poorly understood. The ten-eleven translocation (Tet) family of methylcytosine dioxygenases, which includes Tet1, Tet2, and Tet3 enzymes, has been recently implicated in DNA demethylation. Tet proteins possess 2-oxoglutarate and Fe(II)-dependent oxygenase activity and have been shown to convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) as well as to 5-formylcytosine and 5-carboxylcytosine (Tahiliani et al., 2009; Ito et al., 2010; He et al., 2011; Guo et al., 2011a; Zhang et al., 2013). 5hmC has been proposed to act as an intermediate in either passive demethylation, via disrupting interactions with DNMT1, a DNA methylation maintenance enzyme (Smith and Meissner, 2013), or in active demethylation, involving activation-induced deaminase (AID)/apolipoprotein B mRNA-editing enzyme complex (APOBEC) and the base-excision repair machinery (Guo et al., 2011a, 2011b).

Tet proteins and 5hmC are abundant in the zygote, in embryonic stem cells, and in the brain. Most of the studies on Tet proteins so far have concentrated on their roles in embryonic stem cells (ESCs) and early development. ESCs have relatively high 5hmC content and express Tet1 and Tet2 (Tahiliani et al., 2009; Ito et al., 2010; Williams et al., 2011; Xu et al., 2011; Song et al., 2011; Piccolo et al., 2013). It has been demonstrated that 5hmC and as well as Tet1 are particularly enriched at the transcription start sites and gene bodies of a large number of genes with high CpG content (Williams et al., 2011). However, loss of *Tet1* and *Tet2* and depletion of 5hmC in ESCs does not affect ESC maintenance and pluripotency but leads to subtle differentiation defects (Koh et al., 2011; Dawlaty et al., 2013). Mice deficient in Tet1 or Tet2 are viable, while combined loss of these genes leads to epigenetic abnormalities and partially penetrant perinatal lethality (Dawlaty et al., 2011; Dawlaty et al., 2013).

The abundance of all three Tet proteins as well as 5hmC in mouse brain (Kriaucionis and Heintz, 2009; Szulwach et al., 2011) suggests potential roles of Tet enzymes in postmitotic neurons. Although the functional data regarding neuronal Tet proteins is scarce, recently it was suggested that hydroxylation of 5hmC by Tet1 promotes active DNA demethylation in the adult mouse brain (Guo et al., 2011a). CpG demethylation of the promoters of *Bdnf IX* and *Fgf1B* genes caused by synchronous activation of adult dentate gyrus granule cells (Ma et al., 2009) was shown to be abolished by knocking down endogenous *Tet1* using short-hairpin RNA (Guo et al., 2011a). This study provided the first glimpse into the potential roles of Tet proteins in the nervous system. Recently, MeCP2 (Mellén et al., 2012) and Uhrf2 (Spruijt et al., 2013) were identified as readers of 5hmC in the brain; however, the functional significance of these interactions remains unclear.

A potential connection between neuronal Tet protein function and cognitive processes can be hypothesized following the discovery that (de)methylation of DNA in the brain appears to play a role in learning and memory (Miller and Sweatt, 2007; Miller et al., 2008). Pharmacological inhibition of DNA methylation resulted in defects in synaptic plasticity and memory impairment (Miller et al., 2010), and double knockout of DNMT1 and DNMT3s in the forebrain of mice led to demethylation of a number of neuronal genes and also produced deficits in synaptic plasticity, learning, and memory (Feng et al., 2010). By demonstrating a regulatory role of DNA demethylation in cognitive function (Rudenko and Tsai, 2013), these studies provide the rationale to further study the role of the Tet proteins in the nervous system.

In the current work, we show that the expression of a number of genes is dysregulated in the cortex and hippocampus of Tet1 knockout (Tet1KO) mice. Interestingly, the most prominent category of downregulated genes is comprised of multiple neuronal activity-regulated genes that include *Npas4*, c-*Fos*, *Arc*, *Egr2*, and *Egr4* (Loebrich and Nedivi, 2009; Ebert et al., 2013). We also found that while Tet1KO mice display normal memory formation, they showed specific impairments in extinction learning. Moreover, we show that while hippocampal long-term potentiation was intact in Tet1KO animals, they had abnormally enhanced long-term depression compared to controls. We performed methylation analysis of a key upstream neuronal activity-regulated gene, *Npas4*, and found hypermethylation of the promoter region in Tet1KO animals compared to controls, both in naive mice and after extinction training, which could lead to the reduced expression of *Npas4* and its downstream targets. Our study identifies an important role for Tet1 in regulating the neuronal activity-regulated genes, hippocampal synaptic plasticity, and memory extinction.

RESULTS

Adult Tet1KO Brains Are Morphologically Normal, Have a Significant Reduction in 5hmC, and a Minor Increase in 5mC Levels

Reports of high levels of 5hmC in the CNS genome (Kriaucionis and Heintz, 2009; Szulwach et al., 2011) prompted a search for potential functions for the Tet1 methylcytosine dioxygenase in the mouse brain. We utilized a previously characterized Tet1 knockout (Tet1KO) mouse strain in which exon 4 of *Tet1* is deleted, leading to an out-of-frame fusion of exons 3 and 5 and creating a Tet1 null allele (Dawlaty et al., 2011). Loss of Tet1 mRNA was confirmed by real-time quantitative PCR in cortex and hippocampus (Figure S1A available online). We also quantified all three Tet mRNA levels in hippocampal and cortical tissues from wild-type mice and found that all three Tets are expressed in both hippocampus and cortex (Figure S1B). The presence of all Tet proteins in the CNS may lead to potential compensatory effects caused by the loss of a single Tet family member.

Since Tet proteins are responsible for the conversion of 5mC to 5hmC, we wanted to determine how Tet1 ablation affects 5mC and 5hmC levels in the brain. Global genomic 5mC and 5hmC contents in the hippocampi and cortices of 4-month-old Tet1KO and control Tet1+/+ mice were assessed by immunohistochemistry (Figure 1A) and quantified by liquid chromatography combined with tandem mass spectrometry using multiple reaction monitoring (LC/MS/MS-MRM). We found that deletion of Tet1 led to a relatively minor but significant decrease in global 5hmC levels in the cortex and hippocampus ($p < 0.05$; $p < 0.05$), as well as a subtle and not statistically significant increase in the global levels of 5mC ($p > 0.05$) (Figure 1A). These relatively small overall changes in the global genomic levels of 5hmC and 5mC in the Tet1KO brains are likely to reflect compensatory functions from Tet2 and Tet3, which are also expressed in the brain (Figure S1B).

Anatomical and morphological characterization of the Tet1KO brains did not reveal any significant abnormalities. The number of neurons in different brain areas including the cingulate cortex and hippocampus (Figures 1C, 1D, and data not shown) and the average brain weight (Figure 1E) were unaffected by Tet1 ablation, suggesting that Tet1 is dispensable for normal brain development and/or that Tet2 and Tet3 compensate for the loss of Tet1.

In order to examine more specifically synaptic connectivity in Tet1KO brains, we performed morphological analysis of various brain areas in control and Tet1KO littermate mice (3 + 3 animals, 3 months old) using Synapsin I as a marker of synaptic abundance. As no significant differences in amount or distribution of Synapsin I were found in the cortex and hippocampus of Tet1KO and Tet1+/+ mice ($p > 0.05$; $p > 0.05$; Figure S1C), we conclude that synaptic development remains largely unperturbed by the loss of Tet1.

Tet1 Ablation Does Not Affect Overall Health, Locomotion, Anxiety, and Depression-Related Behaviors

To evaluate potential effects of Tet1 ablation upon behavior of adult mice, we performed a general battery of behavioral tests using 4-month-old Tet1KO and Tet1+/+ male littermate mice (8 to 12 animals per group). None of the animals used in these tests had any overt anatomical or developmental abnormalities (data not shown). We observed normal locomotor behavior in the Tet1KO mice in the open field across all parameters measured ($p > 0.05$; Figure S2A). In addition, parameters characterizing anxiety did not differ significantly between the mutant and control groups ($p > 0.05$; Figure S2B). Tet1KO mice were also indistinguishable from their Tet1+/+ littermates in another

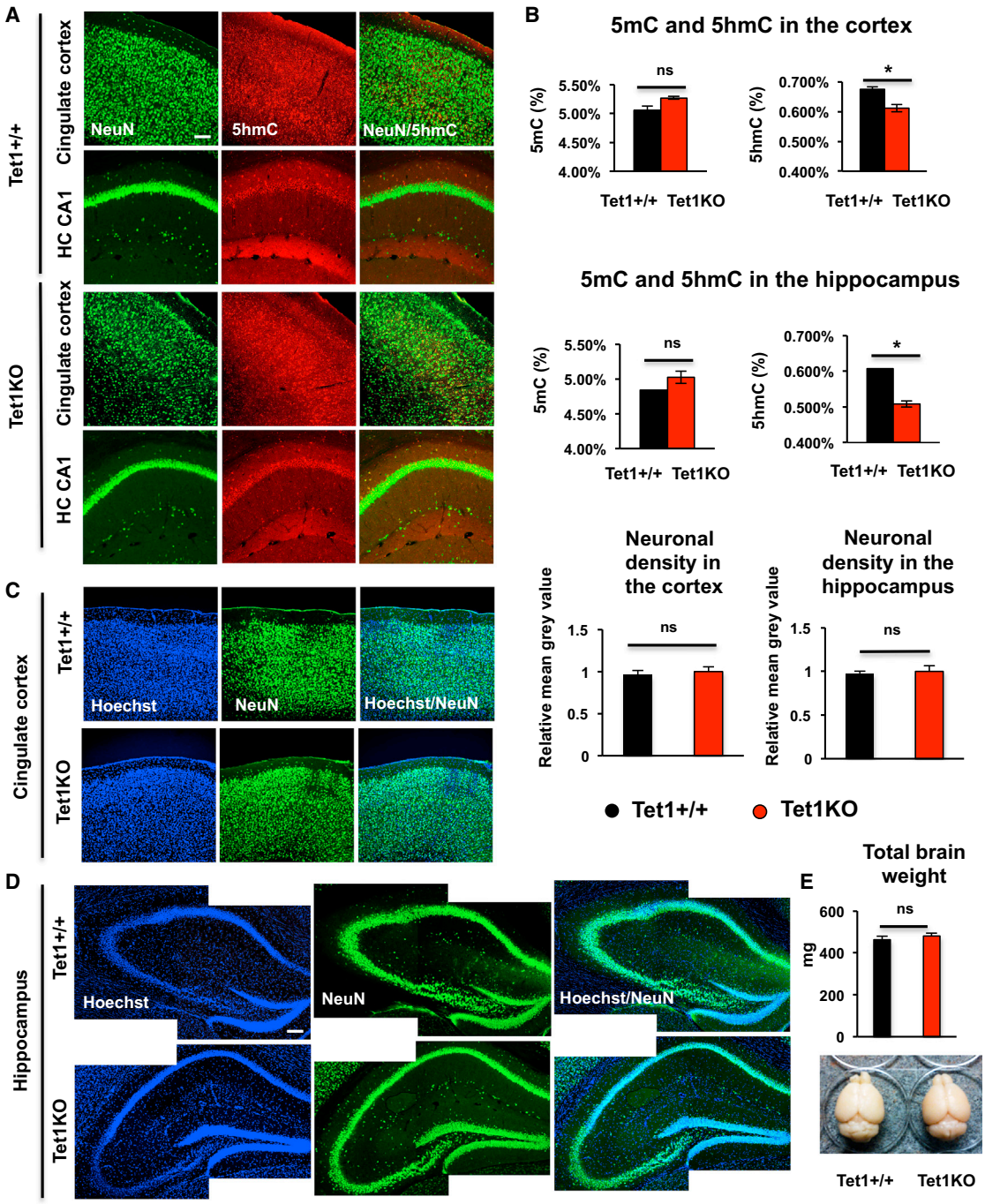

Figure 1. Loss of Tet1 Does Not Affect Brain Size and Morphology but Leads to a Reduction in 5hmC Levels in the Cortex and Hippocampus

(A and B) Global genomic 5hmC levels are significantly reduced, and 5mC levels are slightly but not significantly increased, in the cortex and hippocampus of Tet1KO mice compared to control Tet1+/+ animals. Graphic representation of 5hmC and 5mC levels in the cortex and hippocampus of Tet1+/+ (black) and Tet1KO (red) mice analyzed by liquid chromatography/mass spectrometry multiple reaction monitoring (LC/MS/MS-MRM) (3 + 3 animals). (ns, not significant; *$p < 0.05$; error bars ± SD.) (A) Representative images of the cingulate cortex and hippocampus CA1 are shown. (red, 5hmC; green, NeuN.) (B) Graphs showing the global genomic levels of 5mC and 5hmC in the cortex and hippocampus of Tet1+/+ (black) and Tet1KO (red) mice.

(C–E) Tet1KO mice have normal neuronal number in different brain areas (C and D) and normal total brain weight (E). (blue, Hoechst; green, NeuN; 3 + 3 animals; scale bar, 100 μm; ns, not significant; *$p < 0.05$; **$p < 0.01$; error bars ± SEM.) See also Figure S1.

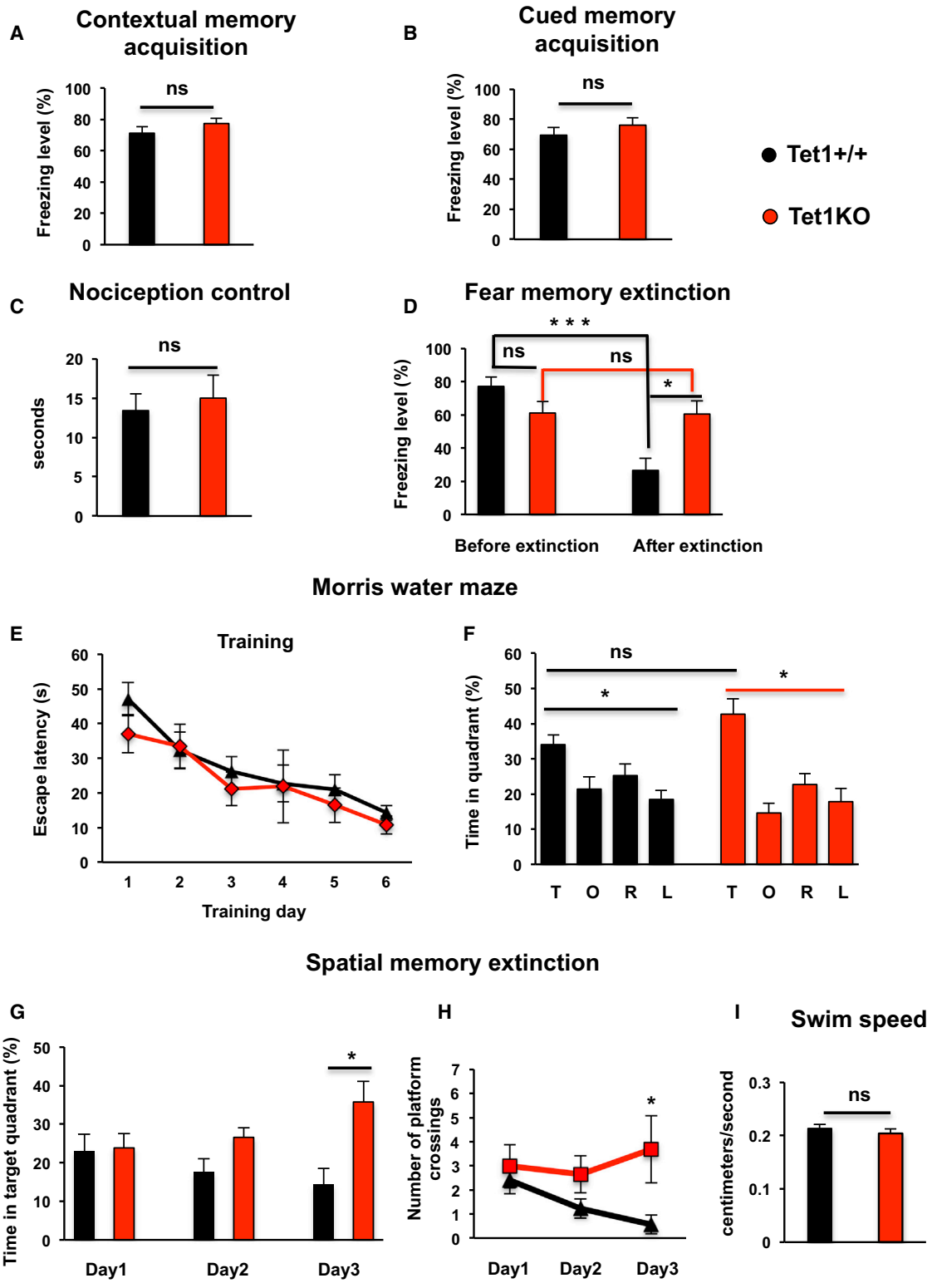

Figure 2. Tet1KO Mice Exhibit Normal Memory Acquisition but Impaired Memory Extinction

(A and B) Tet1KO mice demonstrate normal associative fear memory acquisition in contextual (A) and cued (B) Pavlovian fear conditioning paradigms (auditory cue: 30 s, 20 kHz, 75 db sound pressure level; foot shock: 2 s, 0.8 mA, constant current; 11 + 11 animals).

(C) Tet1KO mice have normal nociception measured by hot plate behavior.

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common test for anxiety-like behavior in rodents, the elevated-plus maze (Dawson and Tricklebank, 1995) (Figure S2C). Using the Porsolt forced swim test (Petit-Demouliere et al., 2005), a measure of depressive-like behavior in the rodents, we also observed no significant differences between the Tet1KO and Tet1+/+ animals ($p > 0.05$; Figure S2D).

Tet1KO Mice Exhibit Normal Acquisition of Fear Memory

As DNA (de)methylation in the brain appears to be important for cognition (Miller et al., 2008, 2010), we wanted to examine hippocampus-dependent learning and memory in Tet1+/+ and Tet1KO animals. To do this, we performed a classical Pavlovian fear conditioning (Phillips and LeDoux, 1992). We observed no difference between the groups in contextual learning ($p > 0.05$; Figure 2A) as well as cued fear memory acquisition ($p > 0.05$; Figure 2B). Hot plate tests showed that there were no differences in nociception between the Tet1KO and Tet1+/+ animals ($p > 0.05$; Figure 2C).

Tet1KO Mice Exhibit Impairment in Memory Extinction

The initial behavioral experiments indicate that associative memory acquisition appears to be unaffected by the loss of Tet1. To examine cognitive functions in more detail, we extended our cognitive evaluation by assessing memory extinction. Memory extinction is a form of inhibitory learning that provides a basis for an adaptive control of cognition and represents one of the key aspects of mental flexibility (Radulovic and Tronson, 2010; Herry et al., 2010; Floresco and Jentsch, 2011). While it has been demonstrated that various stages of memory extinction require the recruitment of widespread brain domains, the molecular mechanisms regulating this process remain unclear (Lattal and Abel, 2001; Lattal et al., 2003; Fischer et al., 2004, 2007; Sananbenesi et al., 2007; Radulovic and Tronson, 2010; Agis-Balboa et al., 2011; Bahari-Javan et al., 2012; Tronson et al. 2012).

In order to test the effects of Tet1 ablation on memory extinction, we performed extinction training using two groups of male littermate Tet1+/+ and Tet1KO mice following contextual fear conditioning as described earlier. Twenty-four hours after fear conditioning, both Tet1+/+ and Tet1KO groups exhibited similar freezing levels (65%–75%) ($p > 0.05$; Figure 2D). After contextual fear memory test, Tet1+/+ and Tet1KO animals were placed into the same conditioning cages for a ''massed'' extinction trial (Cain et al., 2003; Polack et al., 2012). Twenty-four hours later, memory extinction was assessed by scoring freezing events. Interestingly, we found that while control Tet1+/+ mice exhibited robust memory extinction (~20% freezing after extinction training), Tet1KO mutants failed to display any memory extinction and retained an average freezing level of about 60% ($p < 0.01$ control versus Tet1KO; Figure 2D).

To extend cognitive evaluation of the Tet1KO mice, we assessed their hippocampus-dependent spatial reference memory using the Morris water maze test (MWM) (Vorhees and Williams, 2006). Two groups of male littermate Tet1+/+ and Tet1KO mice were subjected to two training trials per day for 6 days, and the escape latency was scored for each trial. Probe trial was conducted 24 hr after the last day of training. We observed no significant differences between the groups during either the training or the probe trials ($p > 0.05$; $p > 0.05$; Figures 2E and 2F). To test spatial memory extinction, we then exposed control and Tet1KO mice used for spatial learning tests to extinction training in the MWM with the same spatial cues but with the platform removed (Zhang et al., 2011). We discovered that while Tet1+/+ mice demonstrated considerable memory extinction as evidenced by their progressively decreasing target quadrant occupancy (from about 35% on the probe trial to about 15% on the last day of extinction training), the Tet1KO animals persevered searching for the platform in the former target quadrant and did not show any decrease in quadrant preference ($p > 0.05$ for Tet1KO; $p < 0.05$ for control versus Tet1KO on day 3; Figure 2G). Additionally, while the Tet1+/+ mice decreased their number of platform crossings from an average 2.8 to an average 0.5, Tet1KO actually increased their number of crossings—from an average of 3 to 3.7 ($p > 0.05$ for Tet1KO and $p < 0.05$ for control versus Tet1KO on day 3; Figure 2H). Control experiments showed a similar swim speed in Tet1+/+ and Tet1KO animals ($p > 0.05$; Figure 2I).

Tet1KO Mice Display Normal Basal Synaptic Transmission and Long-Term Potentiation but Have Abnormally Increased Hippocampal Long-Term Depression

As long-term potentiation (LTP) and long-term depression (LTD) are the critical components of synaptic plasticity, we decided to investigate LTP and LTD in acute hippocampal slices from four pairs of behaviorally naive 6-week-old Tet1+/+ and Tet1KO littermate mice. First, we evaluated basal synaptic transmission in hippocampal slices. The input-output curve was obtained by plotting the slopes of field excitatory postsynaptic potentials (fEPSPs) against fiber volley amplitudes. Presynaptic release probability was assessed by paired-pulse facilitation (PPF) ratio. Our analysis did not show a significant difference in the input-output curve and in PPF between Tet1+/+ and Tet1KO mice ($p > 0.05$; $p > 0.05$; Figures 3A and 3B), indicating normal basal synaptic transmission in Tet1KO mice. In order to evaluate intrinsic neuronal properties, we measured intact presynaptic excitability of hippocampal neurons in control and Tet1KO mice (3 + 3 animals; 5 and 6 slices respectively) and found no significant difference ($p = 0.2848$; Figure 3C).

(D) Tet1KO mice show deficit in fear memory extinction. Graph displays the levels of freezing 24 hr following contextual fear conditioning for Tet1+/+ (black) and Tet1KO (red) animals (before extinction) and 24 hr following massed extinction of contextual freezing (after extinction) (10 + 9 animals).
(E and F) Tet1KO mice show normal spatial reference memory acquisition in the Morris water maze during training (E) and probe trial (F) (time spent in the target [T], opposite [O], right [R], and left [L] quadrants during probe trial).
(G and H) Tet1KO mice show impaired spatial reference memory extinction. (G) Time spent in the target (T) and opposite (O) quadrants during no-platform trials on days 1–3 of extinction training. (H) Number of crossings of the platform area on different days of extinction training
(I) Similar swim speed in Tet1+/+ and Tet1KO mice (10 + 9 animals; ns, not significant; *$p < 0.05$; error bars ± SEM.) See also Figure S2.

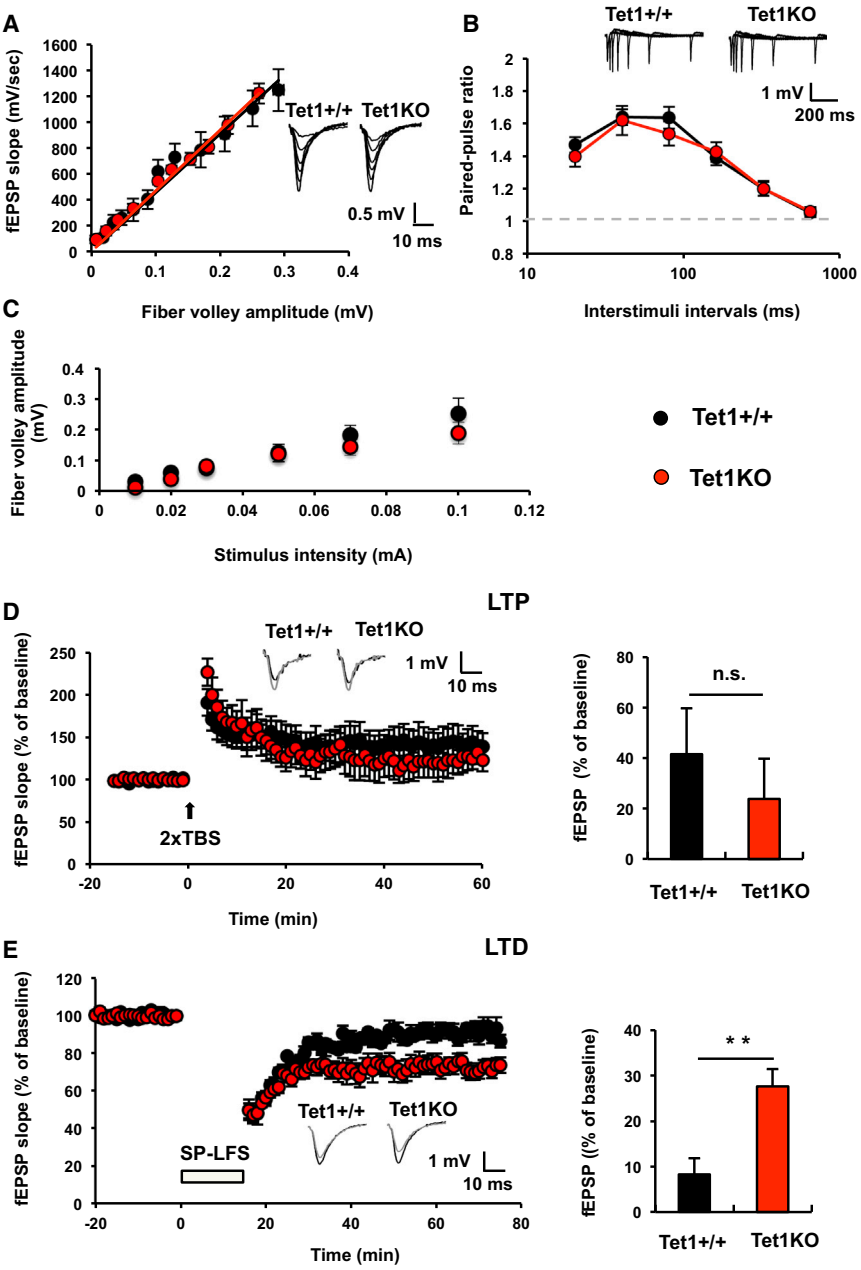

Figure 3. Normal Basal Synaptic Transmission and LTP but Abnormally Enhanced Hippocampal LTD in Tet1KO Mice

(A and B) Tet1 mice demonstrate normal basal synaptic transmission. CA1 fEPSPs evoked by Schaffer collateral pathway stimulation in acute hippocampal slices. (A) The input-output curve was obtained by plotting the slopes of fEPSP against fiber valley amplitudes. (B) PPF ratio was plotted against various interstimulus intervals.

(C) Similar intact presynaptic excitability of hippocampal neurons in Tet1KO mice (red) compared to controls (black). Presynaptic fiber volley amplitude was plotted against various stimulus intensities (3 + 3 animals; 5 and 6 slices).

(D) Normal hippocampal LTP in Tet1KO mice. LTP in CA1 in acute hippocampal slices from 6-week-old Tet1+/+ (black) (four animals, seven slices) and littermate Tet1KO (red) (four animals, six slices) mice evoked by 2× theta-burst stimulation (TBS). fEPSP slope is shown as percentage of baseline. A bar graph shows the average of fEPSP as a percentage of baseline during the last 10 min of recording.

(E) Tet1KO mice exhibit abnormally increased hippocampal LTD. LTD was induced in the Shaffer collateral-CA1 synapses by single-pulse low-frequency stimulation (900 stimuli, 1 Hz) in hippocampal slices from four pairs of behaviorally naive 6-week-old Tet1+/+ (black) and Tet1KO (red) littermate mice (eight slices per group). A bar graph represents the average of fEPSP as a percentage of baseline during the last 10 min of recording (ns, not significant; *$p < 0.05$; **$p < 0.01$; error bars ± SEM.) See also Figure S3.

Next, we examined LTP in the Schaffer collateral-CA1 pathway. CA1 fEPSPs were evoked by Schaffer collateral (SC) stimulation and LTP was induced by two episodes of theta-burst stimulation (TBS) with 10 s intervals. This stimulus induced LTP in both control and mutant mice with a slight trend toward a decreased LTP in Tet1KO mice (control: 141.47% ± 18.18%, Tet1KO: 123.82% ± 15.96%, $p = 0.48$; Figure 3D).

LTD was induced in the Shaffer collateral-CA1 synapses by single-pulse low-frequency stimulation (900 stimuli, 1 Hz). Interestingly, we discovered that while such stimulation was able to weakly induce LTD (91.71% ± 3.51%; Figure 3E) in slices from control Tet1+/+ mice, which is expected considering advanced age of the animals, LTD induction in Tet1KO slices was stronger (72.38% ± 3.74%; Figure 3E) than one would expect from adult mice (Feng et al., 2010). In order to test for potential alterations in metabotropic glutamate receptor (mGluR)-dependent form of LTD in Tet1KO mice, we induced and recorded mGluR-dependent LTD in the slices from three pairs of 3-week-old mice control and Tet1KO littermate mice. Data analysis demonstrated that there was no difference in mGluR-dependent LTD between Tet1KO (73.64% ± 6.34%) and controls (72.49% ± 11.15%) (Figure S3A). As it appears that LTD abnormalities in Tet1KO are confined to NMDAR-dependent LTD, we conducted analysis of expression of various NMDAR subunits in Tet1KO and control brains. We found no differences in expression of any subunits between Tet1KO and Tet1+/+ behaviorally naive mice as well as between the groups of animals that underwent fear conditioning and fear memory extinction training (Figure S3B). This is not surprising, as previous studies have demonstrated abnormal NMDA-dependent LTD in the absence of any changes in NMDAR subunits levels (D'Amelio et al., 2011).

Loss of Tet1 Leads to a Significant Downregulation of Multiple Neuronal Activity-Regulated Genes

To systematically examine whether loss of Tet1 resulted in global alterations in gene expression, we performed microarray analysis using mRNA from the hippocampus and cortex from behaviorally naive littermate Tet1KO and Tet1+/+ mice. These analyses showed that expression of a number of genes was dysregulated in both hippocampus and cortex: 52 genes were upregulated in the hippocampus and 118 in the cortex, while 204 genes were downregulated in the hippocampus and 120 in the cortex (Figure 4A; Tables S1 and Table S2). Interestingly, while the number of genes up- and downregulated in the cortex was similar, about four times more genes were downregulated in the hippocampus of Tet1KO mice compared to control littermates. Analysis of the differentially expressed genes common to both cortex and hippocampus revealed a group of prominent neuronal activity-regulated genes, including *Npas4* and c-*Fos*, while *Egr2*, *Egr4*, and *Arc* were also downregulated in the hippocampus (Figure 4A). Neuronal activity-regulated genes are known to play important roles in a variety of cellular processes including neurotransmission, neuronal plasticity, and learning and memory (Loebrich and Nedivi, 2009). Interestingly, the transcript that displayed one of the strongest levels of downregulation in both cortex and hippocampus of Tet1KO mice was *Npas4* (neuronal PAS domain protein 4) (Figure 4A). This protein was recently shown to regulate a transcriptional program involving neural activity-regulated genes and is critical for cognitive regulation (Ramamoorthi et al., 2011; Coutellier et al., 2012). To confirm the results of our microarray analysis, we performed quantitative real-time PCR using cortical and hippocampal mRNA samples from Tet1+/+ and Tet1KO mice. Based on data from our microarrays, we tested expression levels of *Npas4*, *Arc*, c-*Fos*, *Egr2*, and *Egr4* genes. Results of quantitative real-time PCR experiments confirmed our microarray findings demonstrating a significant decrease in the amount of *Npas4* and c-*Fos* mRNA in the cortex and *Npas4*, *Arc*, c-*Fos*, *Egr2*, and *Egr4* mRNA in hippocampus (Figure 4B).

Next, we examined the protein levels of a representative immediate early gene, c-*Fos*, in the cortex and hippocampus of Tet1+/+ and Tet1KO mice. c-Fos, unlike Npas4, can be readily identified in neurons at baseline levels using immunohistochemistry, which would allow us to examine the spatial distribution of its expression in the brain. The examination of three Tet1KO and three Tet1+/+ brains revealed that levels of c-Fos protein are significantly lower in both the cortex and hippocampus of Tet1KO mice compared to controls (Figure 4C), which is in agreement with the microarray and quantitative real-time PCR findings, while no spatial differences in c-Fos expression were observed (data not shown).

As Tet proteins are responsible for the conversion of 5mC to 5hmC and regulation of the DNA methylation status in various tissues, which may have an effect on chromatin structure and gene expression (Guo et al., 2011b; Branco et al., 2012; Cohen et al., 2011), we hypothesized that downregulation in expression of learning- and memory-related genes in Tet1KO brains may be due to a direct role of Tet1 in the regulation of methylation of these genetic loci. As *Npas4* has been shown to function as a critical upstream regulator of a genetic program that includes other activity-regulated neuronal plasticity genes, such as c-*Fos*, we decided to concentrate upon the analysis of the methylation status of the *Npas4* in the brain of the Tet1+/+ and Tet1KO mice. We therefore performed sodium bisulfite sequencing of the *Npas4* promoter-exon1 junction area, which contains 14 CpGs in the promoter region and 26 CpGs in exon 1. Sodium bisulfite sequencing of DNA from the control brains showed that the *Npas4* promoter-exon 1 junction is methylated in both cortex (~3.5% of CpGs methylated) and hippocampus (~8% of CpGs methylated). We found that the same DNA region was hypermethylated in the Tet1KO mouse cortex (~20%), compared to controls, and it was even more highly methylated in Tet1KO hippocampus (~45%) (Figure 4D). Thus, the loss of Tet1 appears to increase CpG methylation in the promoter-exon 1 region of *Npas4* in the Tet1KO mouse hippocampus and cortex, which may result in its decreased expression. Consistently, applying Gluc-MSqPCR method, we found reduced 5hmC coupled with increased 5mC levels at the promoter region of *Npas4* (Figure S4A) in Tet1KO mice.

Tet1 Loss Causes Downregulation of Neuronal Activity-Regulated Genes and Hypermethylation of *Npas4* Promoter Region after Memory Extinction

There is little data on the molecular mechanisms specifically regulating memory extinction (Lattal et al., 2003; Myers and Davis, 2007; Radulovic and Tronson, 2010). One of the genes that have been demonstrated to be important for extinction is c-*Fos* (Herry and Mons, 2004; Tronson et al., 2009). As expression of a set of neuronal activity-regulated genes was strongly altered in the brains of Tet1KO mice, we hypothesized that such dysregulation may be responsible for memory extinction and synaptic plasticity impairment in Tet1KO animals. Since c-*Fos* and its critical upstream regulator *Npas4* were among a few genes consistently downregulated in both cortex and hippocampus in naive Tet1KO mice, we decided to test their expression after memory extinction training. Six pairs of 4-month-old male Tet1+/+ and Tet1KO littermate mice were subjected to Pavlovian contextual fear conditioning followed by massed memory extinction training as described earlier. The groups of three control and Tet1KO mice were sacrificed 20 min after the training, and mRNA was extracted from hippocampal and cortical tissues to perform gene expression analysis. Another three animals from each group were sacrificed 1 hr after extinction training for protein evaluation.

Quantitative real-time PCR analysis of the samples from postextinction Tet1+/+ and Tet1KO mice showed a significant reduction in the levels of *Npas4* and c-*Fos* transcripts in both hippocampus and cortex. There was roughly 2-fold difference in *Npas4* mRNA levels in both hippocampus and cortex and similar difference in c-*Fos* mRNA ($p < 0.05$; $p < 0.05$; Figure 5A).

In order to evaluate expression and localization of c-Fos and Npas4 proteins in Tet1KO brains after memory extinction, we again used immunohistochemistry. The protein levels were estimated in the hippocampus and cingulate cortex area of Tet1+/+ and Tet1KO mice (3 + 3 animals). These brain regions were chosen as both cingulate cortex and hippocampus have been implicated in contextual memory extinction and cognitive flexibility (Myers and Davis, 2007; Floresco and Jentsch, 2011; Etkin

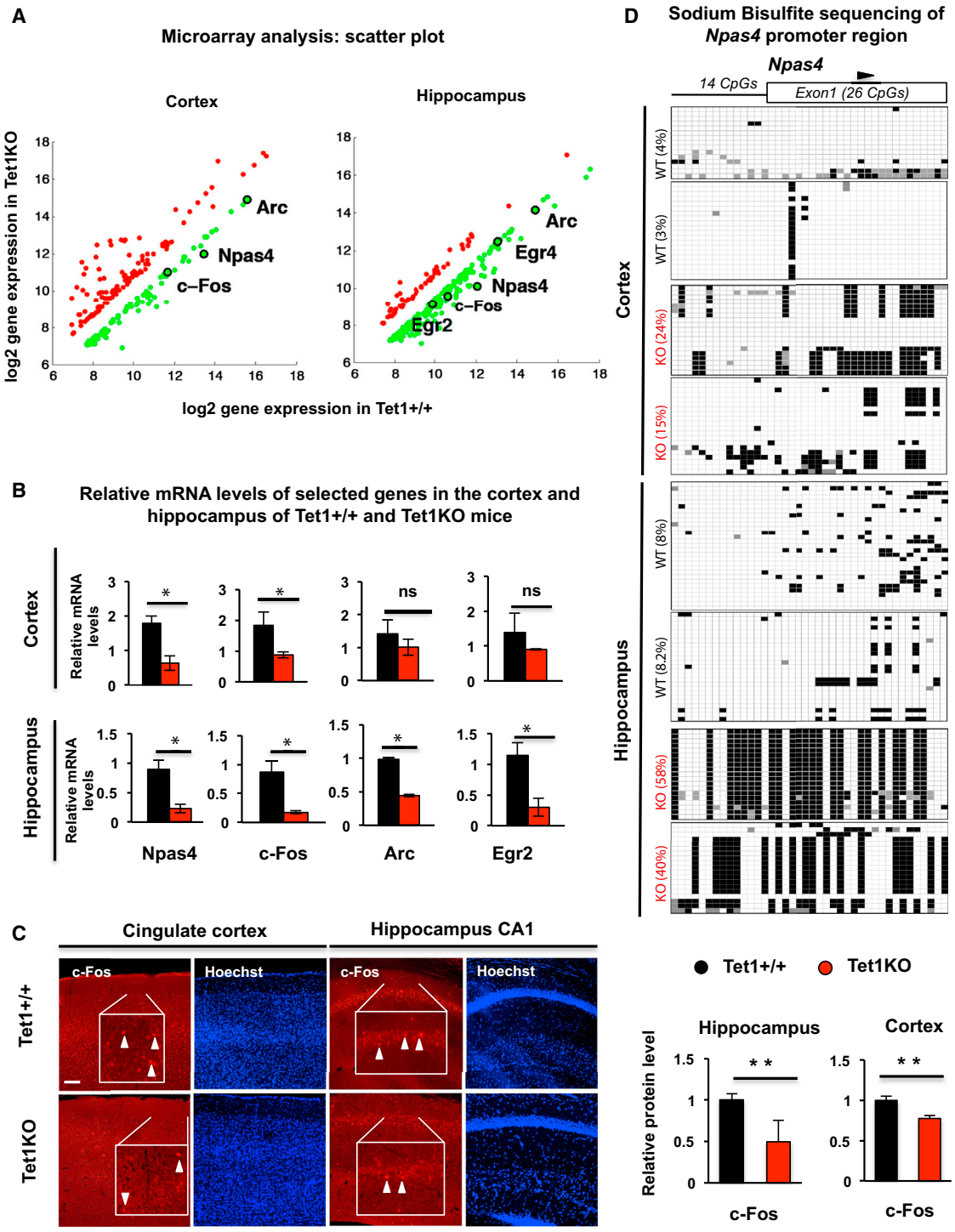

Figure 4. Tet1 Loss Causes Significant Downregulation of Multiple Neuronal Activity-Regulated Genes in the Brain

(A) Microarray analysis of gene expression in the cortex and hippocampus was performed using naive 4-month-old Tet1+/+ and Tet1KO littermate mice (2 + 2 animals). Scatterplot demonstrated that 118 genes were found to be significantly upregulated (red dots) and 120 downregulated (green dots) in the cortex of Tet1KO mice; 52 genes were upregulated (red dots) and 204 downregulated (green dots) in the hippocampus of Tet1KO animals. Set of neural activity-regulated genes including *Npas4*, *c-Fos*, and *Arc* was downregulated in both cortex and hippocampus.

(B) Quantitative real-time PCR was used to validate microarray data (3–5 animals per genotype including those used for microarray analysis, ns, not significant; *$p < 0.05$; error bars ± SD).

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et al., 2011). Examination of three Tet1KO and three control brains revealed that Npas4 and c-Fos protein levels appear to be significantly reduced in Tet1KO mice (Figures 5B and 5C). We failed to detect any differences in the spatial distribution of Npas4 or c-Fos expression between Tet1KO and Tet1+/+ brains following extinction training (Figure 5B and data not shown), suggesting that loss of neuronal Tet1 leads to mostly quantitative rather than spatial brain-region-specific alterations in expression of these genes.

As we observed downregulation of *Npas4* and its target, c-*Fos*, not only in naive mice but also after memory extinction, we wanted to examine the underlying mechanisms. The methylation status of a key upstream neuronal plasticity gene *Npas4* was assessed by sodium bisulfite sequencing after extinction training on the same promoter-exon 1 region studied earlier. Interestingly, methylation analysis showed that postextinction *Npas4* promoter-exon 1 junction remains hypomethylated in both cortex (~8% of CpGs methylated) and hippocampus (~8% of CpGs methylated) in control animals. We found that similarly to naive animals, the promoter region of *Npas4* was hypermethylated in Tet1KO cortex (~25% of CpGs methylated) and hippocampus (~30% of CpGs methylated) after extinction training (Figure 5D). Similarly to naive mice, Gluc-MSqPCR analysis revealed reduced 5hmC levels coupled with increased 5mC levels at the promoter region of *Npas4* (Figure S4A). Such hypermethylation of the promoter area of *Npas4* gene in the Tet1KO brain may explain its decreased expression as well as downregulated expression of its target c-*Fos* during memory extinction.

In order to perform direct comparison of *Npas4* and c-*Fos* expression in control and Tet1KO mice under various experimental conditions, we selected three groups of littermate animals: a behaviorally naive group, a group trained using Pavlovian fear conditioning, and a group that underwent fear memory extinction as described earlier. Quantitative real-time PCR analysis of hippocampal mRNA prepared 20 min after the trainings yielded the following results: consistent with previous data, *Npas4* and c-*Fos* were significantly donwregulated in naive Tet1KO animals compared to controls. Interestingly, Pavlovian fear conditioning increased expression of both genes to similar levels in control and Tet1KO mice. However, extinction training resulted in significant induction of *Npas4* and c-*Fos* expression in Tet1+/+ but not in Tet1KO mice (Figure 5E). To assess whether any of three *Tet* genes are induced upon fear condition or extinction training, we measured Tet1/Tet2/Tet3 mRNA levels in hippocampi of the same groups of animals and found that none of the Tets showed obvious induction (Figure S4B).

DISCUSSION

This study focuses on characterization of adult Tet1KO mice and details the consequences of Tet1 loss in the brain. We found that Tet1 ablation leads to downregulation of a group of neuronal activity-regulated genes in cortex and hippocampus, alterations in synaptic plasticity, and specific cognitive impairment in memory extinction. We also show that the promoter region of a critical upstream factor regulating multiple neuronal activity-regulated genes, *Npas4*, is hypermethylated in Tet1KO mice. The proper control of the methylation status of *Npas4* appears to be important for its expression and for the regulation of its downstream targets, such as c-*Fos*, that are instrumental in mediating synaptic plasticity and cognition. Below, we will discuss the potential consequences of our findings and the questions that remain to be answered.

We should first reiterate that we did not observe any abnormalities in the overall health of Tet1KO mice. This finding is in line with our previous work (Dawlaty et al., 2011) that Tet1 is largely dispensable for embryonic and postnatal development. Our extensive examination of postnatal Tet1KO brains did not reveal any obvious abnormalities (Figure 1, Figure S1C, and data not shown) confirming that the loss of Tet1 does not affect embryonic neurogenesis, neuronal differentiation, and brain development. The absence of severe phenotypic abnormalities following total Tet1 ablation is likely due to the fact that this genetic manipulation did not result in a significant overall increase in 5mC content in the brain (Figure 1B). One obvious explanation for this is that Tet1/Tet2/Tet3 proteins play somewhat redundant roles in the maintenance of appropriate DNA methylation levels and conversion of 5mC to 5hmC in the brain and/or that their roles in normal cellular homeostasis are relatively subtle and cannot be discerned from the examinations performed here.

A thorough examination of various behavioral parameters in Tet1KO mice demonstrated normal exploratory behavior, anxiety, and depression-related behaviors. Moreover, Tet1KO mice also demonstrated normal memory acquisition in Pavlovian fear conditioning and Morris water maze tests, indicating that molecular mechanisms and circuits responsible for contextual fear and spatial reference memory acquisition are not significantly affected. Cognitive tests performed to investigate memory extinction in Tet1KO mice demonstrated impairments in both contextual fear memory extinction and spatial reference memory extinction in these animals.

Examination of the potential neurophysiological basis for memory extinction deficit in Tet1KO by hippocampal slice recordings, demonstrated that while having normal basal synaptic

(C) Analysis of c-Fos showed significant downregulation of its protein in both cortex and hippocampus of naive Tet1KO mice. No differences in c-Fos spatial expression patterns were found between control and Tet1KO animals (white arrowheads point at some of the c-Fos-expressing cells; 3 + 3 animals; ns, not significant; *$p < 0.05$; **$p < 0.01$; error bars ± SEM).

(D) Sodium bisulfite sequencing of the promoter-exon 1 junction of *Npas4* containing total 40 CpGs showed that *Npas4* promoter area is strongly hypermethylated in both cortex and hippocampus (~20% and ~45%, respectively) in naive Tet1KO mice and hypomethylated in controls (~3.5% and ~8%, respectively) (2 + 2 animals). Each of the charts in the figure represents one animal. Each column within the chart represents a single CpG position at the promoter-exon1 junction of *Npas4*. Each row represents a single clone sequenced. A total of 10–20 clones were sequenced for each genotype. White boxes represent unmethylated CpG; black boxes represent methylated CpG; gray boxes represent residues undetermined due to poor sequence quality. Arrowhead denotes position of start codon in exon1. See also Figure S4 and Tables S1 and S2.

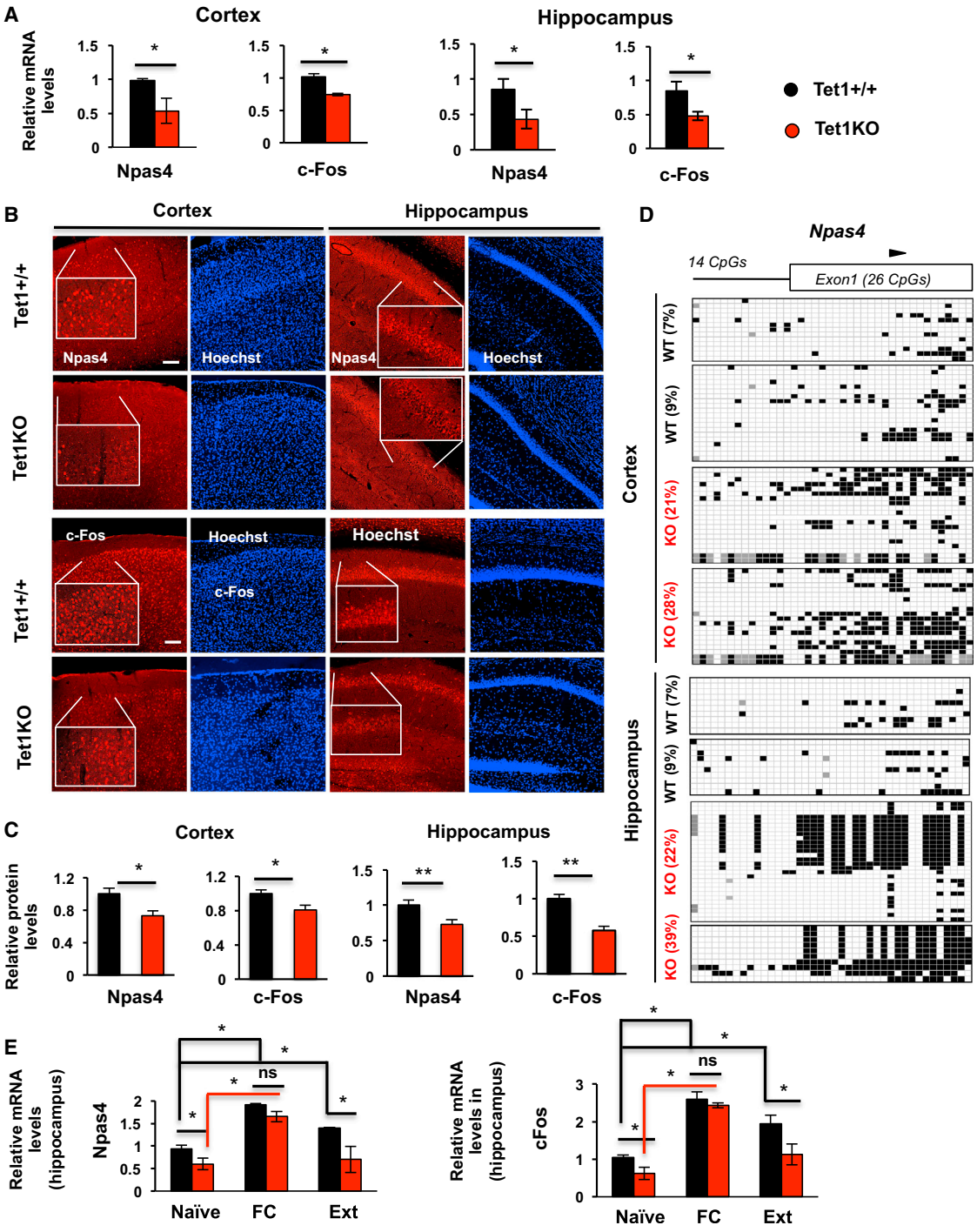

Figure 5. Tet1KO Mice Exhibit Downregulation of Neuronal Activity-Regulated Genes and Hypermethylation of *Npas4* Promoter after Extinction Training

(A–C) mRNA and protein levels of several neuronal activity-regulated genes are decreased in the cortex and hippocampus of Tet1KO mice after memory extinction training. Two groups of male littermate Tet1+/+ and Tet1KO mice underwent contextual fear conditioning (context chamber + 2 s, 0.8 mA constant current shock) followed by massed fear memory extinction training (21 min of context exploration). Twenty-four hours later, mice were placed in the same chamber and given 3 min exploration time to induce extinction memory retrieval. Mice were sacrificed 20 min (3 + 3 animals) and 1 hr (3 + 3 animals) after retrieval for mRNA and protein analysis respectively. (A) Relative mRNA levels of Npas4 and c-Fos in the cortex and hippocampus of Tet1+/+ (black) and Tet1KO (red) are shown. (Three animals used per genotype, ns, not significant; *$p < 0.05$; error bars ± SD.) (B) Representative images of Npas4 and c-Fos protein expression in the cortex and hippocampus Tet1+/+ and Tet1KO mice are shown. (C) Relative protein levels of Npas4 and c-Fos in the cortex and hippocampus are shown. Note

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transmission, presynaptic excitability, and Schaffer collateral-CA1 LTP, Tet1KO mice exhibited an abnormally enhanced LTD. Although we are unsure about the molecular mechanisms underlying LTD abnormalities in Tet1KO mice, it is feasible that consistent downregulation of some of the plasticity-related neuronal genes observed in Tet1KO brain may underlie such impairment. As *Npas4*, c-*Fos*, and other genes can mediate neuronal plasticity at multiple levels (Loebrich and Nedivi, 2009; Alberini, 2009; Lin et al., 2008; Mamiya et al., 2009; West and Greenberg, 2011; Ebert et al., 2013), their downregulation may affect proper function of various components of LTD machinery. For example, it has been shown that LTD is regulated by the AMPA receptor trafficking (Liu and Cull-Candy, 2000; Clem and Huganir, 2010). As we observed misexpression of *Arc* in Tet1KO mice, it is tempting to speculate that LTD abnormality in Tet1KO animals may be at least partially a result of an impairment in Arc-dependent AMPA receptor trafficking (Keifer et al., 2008; Loebrich and Nedivi, 2009). Interestingly, it has been shown that disruption of AMPA receptor endocytosis also impairs extinction, but not acquisition, of learned fear (Dalton et al., 2008). A number of previous studies demonstrated a connection between LTD and memory extinction (Ryu et al., 2008; Dalton et al., 2008; Tsetsenis et al., 2011; Kim et al., 2011). It was even proposed that LTD may represent a synaptic mechanism facilitating selective manipulation of an established memory, while leaving intact the capacity to form new memory (Dalton et al., 2008). Tet1KO mice provide a valuable genetic model for further investigation of the precise mechanistic connection between LTD and memory extinction.

In this study, our genome-wide transcriptional profiling of the cortex and hippocampus of naive control and Tet1KO littermate animals showed that, while approximately the same number of genes was up- and downregulated in the cortex of Tet1KO mice, the majority of altered genes in the Tet1KO hippocampus were downregulated (Figure 4A; Tables S1 and S2). An analysis of the dysregulated genes showed that a group of neuronal activity-regulated genes, including *Npas4*, c-*Fos*, *Arc*, *Egr2*, and *Egr4*, were downregulated in the hippocampus and that *Npas4*, c-*Fos*, and *Arc* also showed downregulation in the cortex. We should note that the key upstream regulatory gene *Npas4* demonstrated the highest levels of dysregulation among all the genes analyzed in Tet1KO mice (Figure 4A). Sodium bisulfite sequencing revealed hypermethylation of the promoter-exon 1 junction of *Npas4* in both cortex and hippocampus of Tet1KO mice compared to control animals. Such hypermethylation provides a likely mechanism for the suppression of *Npas4* the Tet1KO mouse brain, as well as for the downregulation of Npas4 target genes, including c-*Fos*.

We also found that both mRNA and protein levels of Npas4 and c-Fos were downregulated in the cortex and hippocampus of Tet1KO mice compared to controls following memory extinction training. Postextinction methylation analysis of *Npas4* promoter region indicated that its methylation remained low in the cortex and hippocampus in wild-type mice but stayed at much higher levels in Tet1KO animals, strongly correlating with *Npas4* expression. We should note that locus-specific methylation and hydroxymethylation analysis in this study was performed using promoter areas of specific genes. However, we realize that methylation and hydroxymethylation of gene body as well as various distant regulatory elements may also contribute to gene expression (Bergman and Cedar, 2013). Future studies aimed at genome-wide methylation and hydroxymethylation analysis of the brains of Tet-deficient mice may provide a more comprehensive coverage of multiple methylation domains regulating expressions of specific genes.

One plausible explanation for extinction impairment observed in Tet1KO mice would be that the memory extinction paradigm does not provide a sufficiently strong stimulus to overcome Tet1 deficiency. However, a stronger stimulation, such as Pavlovian fear conditioning employed during memory acquisition, may recruit additional transcriptional machinery, including other Tet proteins, thus compensating for the lack of Tet1 (Figure S5). In favor of such hypothesis is the result of our direct comparison of *Npas4* and c-*Fos* expression in control and Tet1KO mice under various experimental conditions (Figure 5E). Further studies using double and triple Tet knockouts should help us better understand the relative contribution and potential co-operation of different Tet proteins in various aspects of activity-driven chromatin regulation, cognitive behavior, and plasticity. It has recently been shown that Tet1 promotes 5mC hydroxylation and active DNA demethylation in the adult mouse brain. Tet1 is both necessary and sufficient for neuronal activity-induced DNA demethylation of the *Bdnf IX* and *Fgf1B* promoters in the dentate gyrus (Guo et al., 2011a, 2011b). It remains to be seen whether such region- and gene-specific activity-dependent DNA demethylation occurs in a more general fashion in the mammalian brain, and whether Tet1 activity is critical. Our findings do not contradict the hypothesis of a key role of Tet1 in activity-induced DNA demethylation, but they also suggest a maintenance-type role for neuronal Tet1, perhaps protecting locus-specific DNA regions from excessive methylation (Williams et al., 2012). We propose that Tet1 plays an important role in maintaining consistent hypomethylation of specific DNA regions including promoter areas of at least some of the neuronal activity-regulated genes. Such hypomethylation may

significant downregulation of both mRNA and protein levels of Npas4 and c-Fos in both cortex and hippocampus of Tet1KO mice after memory extinction (three animals used per genotype; ns, not significant; *$p < 0.05$; error bars ± SD).

(D) Sodium bisulfite sequencing of the promoter-exon 1 junction of *Npas4* showed that this area is hypermethylated in Tet1KO cortex (~24.5% of CpG methylated) and hippocampus (~30.5% of CpGs methylated) compared to control cortex (~8% of CpGs methylated) and hippocampus (~8% CpGs methylated) (2 + 2 animals). Each of the charts in the figure represents one animal. Each column within the chart represents a single CpG position at the promoter-exon1 junction of *Npas4*. Each row represents a single clone sequenced. A total of 10–20 clones were sequenced for each genotype. White boxes represent unmethylated CpG; black boxes represent methylated CpG; gray boxes represent residues undetermined due to a poor sequence quality. Arrowhead denotes position of start codon in exon1.

(E) Direct comparison of *Npas4* and c-*Fos* expression levels in the hippocampus of control and Tet1KO mice in various experimental conditions: in naive animals, after Pavlovian fear conditioning/fear memory acquisition (20 min after fear memory retrieval) and after fear memory extinction training (20 min after extinction memory retrieval). (3 + 3 animals; ns, not significant; *$p < 0.05$; **$p < 0.01$; error bars ± SD.) See also Figure S5.

be important in keeping specific promoters poised for rapid transcriptional activation. This in turn will allow an increased flexibility in transcriptional regulation that may serve as a basis for various cognitive flexibility aspects including memory extinction. Interestingly, we also discovered that all three Tet proteins in the mouse brain did not show induction after Pavlovian fear conditioning and fear memory extinction training. This may suggest that expression of Tet genes is not activity regulated. However, it is also feasible that our behavioral paradigms are not sufficient to facilitate Tet induction or that Tet induction kinetics may follow a relatively slow course.

Based on our findings, we propose that neuronal Tet1 is critical for memory extinction, regulating expression of key neuronal activity-regulated genes and neuronal plasticity. Future examination of other aspects of cognitive flexibility, such as extinction of cued fear memory and reversal learning, as well as further evaluation of different cognitive manifestations, may provide additional insights into the nature of cognitive abnormalities in Tet1KO mice.

Our data demonstrating a role of neuronal Tet1 in memory extinction may have important clinical implications. Posttraumatic stress disorder (PTSD) is a common disorder caused by traumatic psychological events and characterized by an individual re-experiencing the original trauma and experiencing clinically significant distress or impairments in functioning (American Psychiatric Association, 2000, DSM-IV-TR; Porter and Kaplan, 2011, Merck Manual of Diagnosis and Therapy). Based on our findings, Tet1 may represent a potentially exciting molecular target for PTSD therapy. Future research on Tet1, as well as of the other members of the Tet family, may contribute significantly to our understanding of the fundamental mechanisms of memory extinction as well as provide potential treatment for disorders such as PTSD.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed according to the Guide for the Care and Use of Laboratory Animals and were approved by the National Institutes of Health and the Committee on Animal Care at the Massachusetts Institute of Technology (Cambridge, MA, USA). Tet1KO and Tet1+/+ used in the study were generated as reported previously (Dawlaty et al., 2011).

Behavior

Open-field, fear conditioning, and Morris water maze were performed as previously described (Carlén et al., 2012; Gräff et al., 2012) with minor modifications. Elevated plus-maze was performed as previously described (David et al., 2009) with minor modifications. Memory extinction: after contextual fear memory test, Tet1+/+ and Tet1KO groups of mice were placed into the same conditioning chambers for a "massed" fear memory extinction trial (Cain et al., 2003; Polack et al., 2012). Spatial memory extinction was performed after learning in the Morris water maze (MWM) as in Zhang et al. (2011).

Slice Electrophysiology

Transverse hippocampal slices were prepared from 6-week-old and 3- to 4-month-old male littermate mice. CA1 field excitatory postsynaptic potentials evoked by Schaffer collateral pathway stimulation and measured as described in Deng et al. (2011). LTP was induced by four episodes of theta burst stimulation (TBS) with 10 s intervals, and TBS consisted of ten brief bursts of stimuli delivered at 5 Hz; each burst contains four pulses at 100 Hz. mGluR-dependent LTD was induced by treatment of mGluRI agonist DHPG for 10 min, then recorded for an additional hour.

Quantification of 5mC and 5hmC

Quantification of global 5hmC and 5mC was performed as previously described in Dawlaty et al. (2013). Liquid-chromatography combined with tandem mass spectrometry using multiple reaction monitoring (LC-MS/MS-MRM) was used to quantify 5hmC and 5mC levels in the DNA extracted from the cortex and hippocampus of 4-month-old littermate male Tet1+/+ (control) and Tet1KO (mutant) mice (3 + 3 animals).

Microarrays and Quantitative Real-Time PCR

Total RNA was isolated from the cortex and hippocampus of naive Tet1+/+ and Tet1KO male littermate mice (2 + 2 animals; independent samples). Microarray experiments and their analysis was performed as in Dawlaty et al. (2011) and detailed in Supplemental Experimental Procedures. For quantitative real-time PCR, hippocampal and cortical tissues were dissected from the male littermate Tet1+/+ and Tet1KO mice and quantitative real-time PCR was performed essentially as in Gräff et al. (2012). Primers used are outlined in Supplemental Experimental Procedures.

Sodium Bisulfite Sequencing

A total of 2 μg of DNA extracted from cortex or hippocampus of Tet1+/+ and Tet1KO mice (2 + 2 animals) was treated with sodium bisulfite using EpiTect kit (QIAGEN). *Npas4* promoter-exon 1 junction was amplified using the following nested primers: external forward: 5′-GTAAATTGGTAGAGGATTAAGTTTTTTTTTATTTTTTG-3′; external reverse: 5′-TATCTCACACAAATCCAATACTAAAACTATC-3′; internal forward: 5′-TTTTGTTAAGGGTTTTAGATTATTTTAATTTATGTATTG-3′; internal reverse: 5′-AACCCAAACTACTCACCTCCAAC-3′.

PCR product (700 bp) was cloned into pcr2.1 vector TA-cloning vector and sequenced with M13rev primers. A total of 10–20 clones were analyzed per sample. Methylation status of *Npas4* promoter-exon 1 junction was displayed in the form of a grid with columns representing each of the 40 CpG dinucleotides present in this region and each row representing individual clones sequenced.

Statistics

Paired Student's t tests, one-way ANOVA, and two-way ANOVA followed by Bonferroni post hoc test were used where appropriate. The graphs represent mean values with error bars representing SEM or SD. The statistical analysis was performed using GraphPad Prism software (GraphPad Software).

Detailed description of the experimental procedures and reagents is available in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for gene array data sets reported in this paper is GSE48789.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.08.003.

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