Abstract

5-hydroxymethylcytosine (5hmC) is a recently re-discovered transient intermediate in the active demethylation pathway that also appears to play an independent role in modulating gene function. Epigenetic marks, particularly 5-methylcytosine, have been widely studied in relation to stress-related disorders given the long-lasting effect that stress has on these marks. 5hmC is a good candidate for involvement in the etiology of these disorders given its elevated concentration in mammalian neurons, its dynamic regulation during development of the central nervous system, and its high variability among individuals. Although we are unaware of any studies published to date examining 5hmC profiles in human subjects who have developed a psychiatric disorder after a life stressor, there is emerging evidence from the animal literature that 5hmC profiles are altered in the context of fear-conditioning paradigms and stress exposure, suggesting a possible role for 5hmC in the biological underpinnings of stress-related disorders. In this review, the authors examine the available approaches for profiling 5hmC and describe their advantages and disadvantages as well as discuss the studies published thus far investigating 5hmC in the context of fear-related learning and stress exposure in animals. The authors also highlight the global versus locus-specific regulation of 5hmC in these studies. Finally, the limitations of the current studies and their implications are discussed.

Key words: 5-hydroxymethylcytosine; 5hmC; 5hmC detection methods; stress; fear-related learning

Introduction

Initially identified in mammalian DNA in 1972 [1], and ‘re-discovered’ in 2009 after its identification in post-mitotic neurons [2], 5-hydroxymethylcytosine (5hmC) is an oxidized derivative of 5-methylcytosine (5mC). 5hmC was first identified to serve as an intermediate during active DNA demethylation processes but recent work suggests that it also has a unique functional role on its own. During active DNA demethylation, 5hmC is catalyzed by the oxidation of 5mC by the ten–eleven translocation (TET) family of DNA hydroxylases (Tet1, Tet2, and Tet3) [3]. 5hmC is further oxidized by TET enzymes into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) and is then converted back to an unmodified C through nucleotide and/or base excision repair pathways. Multiple pieces of evidence suggest that 5hmC (as well as the other oxidized nucleotides generated by TET enzymes), has an independent function beyond a transient step in active DNA demethylation, including that it is relatively stable, it has non-random and unique distribution in the genome [4], and it has been shown to preferentially bind to specific proteins as compared to 5mC [5]. Additionally, there is evidence from...
biophysical studies that 5hmC has an influence on base-pairing and DNA structure [6].

Although 5hmC can be detected in the genomic DNA of all cells at an overall level of ~10%, the highest concentration is found in mammalian post-mitotic neurons in contrast to 5mC, which is found in relatively stable concentrations throughout somatic tissues [7]. 5hmC is enriched within distal cis-regulatory elements, gene bodies and euchromatin, where it appears to be a predominantly active epigenetic mark, as it positively correlates with gene transcript levels [8]. This is in contrast to 5mC, which has generally been associated with gene repression via the blockade of transcription factor binding and recruitment of repressor proteins that can condense chromatin [9]. Multiple studies have mapped genome-wide 5hmC in healthy human subjects and mice of various ages in the cerebrum and cerebellum. Within the mouse frontal cortex, total 5hmC levels increase from fetal stages to adulthood. Additionally, neuronal and non-neuronal cells display differential 5hmC profiles with enrichment of this mark in neuronal and astrocyte-specific gene bodies starting at fetal stages and persisting into adulthood, suggesting cell type-specific regulation in utero [10]. 5hmC also has been shown to increase with age in the human cerebrum until 1 year of age in white matter and 22 years of age in grey matter, after which time it reaches a steady state [11]. In contrast, 5mC remains relatively stable with age in white matter and shows a moderate increase with age in grey matter. Another study demonstrated greater inter-individual variability of 5hmC than 5mC, enrichment of 5hmC in genes related to neurodevelopment, and differential 5hmC profiles in males and females [12]. Moreover, Lunnun and colleagues found overall increased levels of 5hmC in the cerebrum, as compared with the prefrontal cortex, enrichment in CpG islands and gene bodies in loci showing the greatest differences between brain regions, and high inter-individual variability in a subset of loci [13]. These features of 5hmC distribution across the genome and lifespan as well as its association with gene regulation strongly suggest an independent function beyond a transient step in active DNA demethylation, especially in the nervous system.

**Detection methods for 5hmC**

Profiling 5hmC by sensitive, accurate, and reliable methods that can distinguish 5hmC from 5mC (not the case for traditional bisulfite conversion) is essential for understanding its function. Detection methods for 5hmC include those with global, low, and single nucleotide resolution. Commonly used techniques are discussed here, while the advantages and disadvantages of each method can be found in Table 1. Global techniques assess the overall 5hmC level of cells or tissues and include antibody-based DNA dot-blot, immunohistochemistry (IHC), thin layer chromatography (TLC), and high performance liquid chromatography mass spectrometry (HPLC-MS) [2, 14]. In DNA dot-blot, genomic DNA is immobilized on a membrane and probed with a 5hmC-specific antibody. IHC involves use of an antibody specific for 5hmC to stain fixed cells or tissue sections. In TLC, DNA that has been digested by a restriction enzyme specific for 5hmC, such as Msp I, is labelled at the ends with 32P, hydrolyzed and then separated onto TLC plates. The final global profiling technique is HPLC-MS, which involves resolving hydroxymethylated nucleosides by liquid chromatography followed by detection using mass spectrometry. Overall, these methods assess the average of 5hmC of a population of cells or of a tissue and represent mainly large genomic regions, where this mark is highly enriched. Low-resolution methods give an average level of 5hmC within 200–300 bp fragments that allow us to refine the location of 5hmC. They include enrichment techniques, such as hydroxymethylated DNA immunoprecipitation (hMeDIP), cysteine-5-methylensulfonate (CMS) immunoprecipitation, hydroxymethyl-selective biotin labeling and capture, and J-binding protein 1 (JBP1) 5hmC pull-down as well as 5hmC-sensitive enzymatic digestion [15]. In hMeDIP, DNA is fragmented and incubated with an antibody specific to 5hmC. Following washing of the beads to remove non-specific binding, 5hmC immuno-precipitated (IP) DNA fragments are purified and can then be compared to input DNA (i.e. no-IP). Several 5hmC antibody-based approaches are commercially available with varying degrees of sensitivity and specificity. Thus, it is critical to assess 5hmC antibody specificity prior to hMeDIP and other antibody-based approaches (even for new antibody batches). Specificity is often confirmed via dot-blot, where the presence or absence of antibody binding is assessed for PCR products in which all cytosines are unmodified, methylated, or hydroxymethylated. Synthetic oligonucleotides containing 5mC or 5hmC are commercially available or can be synthesized via PCR employing methyl or hydroxymethyl dCTP such that all cytosines in the amplified product are methylated or hydroxymethylated, respectively. As a true negative control, it would be of significance to validate 5hmC antibody specificity in mouse embryonic stem cells (ESCs) lacking Tet 1, 2, and 3, which have no 5hmC as measured by mass spectrometry [16, 17]. However, the authors are unaware of any validation of 5hmC antibodies in these triple knockout (KO) ESCs. Due to the redundant activity of Tet enzymes, constitutive KO of Tet1 or Tet1 and Tet2 does not result in loss of 5hmC from the genome and, therefore, the published work in other Tet KO animals discussed below are insufficient as negative controls for validation of 5hmC antibody specificity. To avoid the issues of 5hmC antibody specificity, CMS immunoprecipitation relies on the conversion of 5hmC to the modified base CMS, followed by immunoprecipitation with an antibody against CMS. During hydroxymethyl-selective biotin labeling and capture, a glucose that has been modified by azide is transferred onto 5hmC and then selectively chemically labelled by attachment of a biotin tag. In JBP1 5hmC pull-down, epoxy-modified magnetic beads are covalently bound to JBP1, a protein that interacts with β-gluc-5hmC, and used to pull down 5hmC that has been glycosylated using T4 β-glucosyltransferase. Overall, these pull-down techniques give us only a semi-quantitative level of 5hmC at low resolution where the actual loci and levels of 5hmC per site remain unknown.

Methods with near single nucleotide resolution include approaches employing 5hmC-dependent restriction enzymes (e.g. MspI and AbaSI) in combination with glycosylation, although such approaches are dependent on specific sequence requirements and result in reduced representation of the genome [18]. Advancement of these methods was recently published utilizing the PouRts1 I restriction endonuclease in combination with Seal-based chemical labelling for specific isolation of 5hmC-containing DNA fragments following PouRts1 I digestion (Pvu-Seal-Seq), enabling sensitive genome-wide profiling of 5hmC via next-generation sequencing [19]. Although still debated, the gold standards for single nucleotide resolution 5hmC profiling are based on modifications of standard bisulfite sequencing [20]. Oxidative bisulfite (oxBS) conversion employs potassium permanganate to chemically oxidize 5hmC to 5fC, which is then susceptible to bisulfite conversion to uracil, while 5mC and C remain unmodified [21]. Tet-Assisted Bisulfite (TAB) conversion involves the protection of 5hmC by glycosylation from oxidation.
recombinant Tet1-mediated oxidation such that 5mC is oxidized to 5caC without conversion of glycosylated 5hmC [22]. During subsequent standard bisulfite conversion, C and 5caC are converted to uracil while 5hmC remains unmodified. In both of these methods, 5hmC is then characterized via sequencing (oxBS-seq, TAB-seq) or array-based methods. Despite advances in single-base resolution profiling, further development has suffered because of the high cost of whole-genome sequencing; and therefore, these techniques have not been fully utilized by the epigenetic community at large.

| Type of detection | Method | Advantages                                                                 | Disadvantages                                                                                                                                 |
|-------------------|--------|----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| Global            | Antibody-based DNA dot-blot | • Rapid, simple, inexpensive  
• Able to adapt to ELISA format for relative quantification | • Semi-quantitative at best  
• Usually requires microgram quantities of starting DNA  
• Affected by any non-specific antibody binding  
• Requires stringent 5hmC antibody validation  
• Semi-quantitative at best  
• Quite sensitive to sample preparation  
• Affected by any non-specific antibody binding  
• Requires stringent 5hmC antibody validation  
• Not quantitative by itself  
• Radioactive isotopes are needed  
• Requires large quantity of starting DNA |
|                   | IHC    | • Able to visualize 5hmC at the level of a single cell, metaphase chromosomes, and across a tissue sample | |
|                   | TLC    | • 5hmC can be analyzed in different sequence contexts using different enzymes  
• Total 5hmC can be quantified via nearest neighbor analysis | |
|                   | HPLC-MS | • Most accurate method for global quantification of 5hmC  
• Sensitive and accurate  
• Quantitative with the use of reference compounds | • Relatively costly  
• Necessitates expert knowledge, advanced equipment, and large quantities of DNA |
| Locus specific    | hMeDIP | • Does not require the use of specialized equipment | • Regions with high density may be overrepresented, while those with low 5hmC density may be underrepresented  
• Enriches for CA and CT repeats  
• Affected by any non-specific antibody binding  
• Requires stringent 5hmC antibody validation |
|                   | CMS immunoprecipitation | • May be completed in 3 days  
• Less dependent on 5hmC density than 5-HmeDIP | • Antiserum against CMS is not commercially available |
|                   | Hydroxymethyl-selective biotin labeling and capture | • Highly sensitive and efficient | • Labeling and capture efficiency and specificity is significantly reduced by low starting material  
• JBP1 protein is not commercially available  
• Requires up to 2 weeks |
|                   | JBP1 5hmC pull-down | • All components are readily available or can be rapidly synthesized | |
| Single nucleotide | Restriction enzyme-based approaches (e.g. Reduced Representation 5hmC Profiling using MspI, Aba-seq using AbaSI) | • Highly reproducible data | • Requires specific sequence context  
• Reduced representation of all cytosines |
|                   | Pvu-Seal-Seq | • High sensitivity  
• Detection of low abundance 5hmC loci with reduced sequencing coverage | • Cannot quantify absolute levels of 5hmC  
• Restriction digestion increases risk of false positives |
|                   | oxBS conversion | • Protocol can be completed in 2 days  
• The agent used to chemically oxidize 5hmC is highly selective | • DNA damage and degradation by chemical oxidation and repeated bisulfite treatments needed for deamination  
• Requires comparison to standard bisulfite sequencing for single-base resolution 5hmC profiles  
• Requires deep sequencing coverage for high-confidence 5hmC identification |
|                   | C sequenced as T  
SmC sequenced as C  
5hmC sequenced as T | | |
|                   | TAB conversion | • Treatment involves less DNA damage than oxBS | • Requires highly active TET enzymes, which are unstable, difficult to purify, and expensive  
• βGT is inefficient at glycosylating CpG dinucleotides, where 5hmC usually occurs  
• Requires deep sequencing coverage for high-confidence 5hmC identification |
|                   | C sequenced as T  
5mC sequenced as T  
5hmC sequenced as C | | |
However, targeted oxBS-seq and TAB-seq employing custom DNA capture techniques will enable low cost single base resolution mapping of ShmC. With the advent of nanopore and Pacific Biosciences Single Molecule Real Time sequencing (not discussed in Table 1), the ability to identify multiple DNA modifications simultaneously in a given sequencing run also provides exciting avenues for future research, although these technologies are still undergoing rapid development. The authors are unaware of any studies that have directly compared multiple ShmC profiling methods, so method selection must currently be based on the quantity of input DNA, resolution of profiling required, availability of reagents and equipment, as well as the cost.

ShmC and stress
The abundance of ShmC within the brain, its dynamic regulation during development of the central nervous system, and its high inter-individual variability renders it a particularly interesting candidate for involvement in neurological and psychiatric disorders. In both human and animal models, studies have shown differences in ShmC between cases and controls in multiple sclerosis [23], neurodegenerative diseases [24–26], and neuropsychiatric disorders such as autism spectrum disorder [27, 28] and psychosis [29–31]. Although a large body of literature exploring the relationship between 5mC and stress-related psychiatric disorders currently exists given the long-lasting changes that stress can induce on 5mC profiles, the authors are unaware of any studies to date that have profiled the hydroxymethylome in the brains or other tissues of human subjects with psychiatric disorders related to trauma exposure. However, there is a developing animal literature assessing the impact of fear-learning paradigms as well as stress exposure on ShmC profiles.

Stress may be defined as any stimulus that challenges one’s integrity or function and leads to the release of chemical mediators, including corticotropin-releasing hormone and glucocorticoids, in the limbic-hypothalamic-pituitary (LHPA) axis [32]. This process, in turn, leads to changes in neurotransmission, synaptic plasticity, and neural architecture of brain regions that play critical functions in the LHPA axis, including the prefrontal cortex, hippocampus, and amygdala. One way in which psycho-social stressors, such as child abuse or neglect and exposure to traumatic events, may mediate long-term changes in neural architecture is through epigenetic mechanisms such as the well-documented role of DNA methylation in clinical and pre-clinical models of stress-related psychopathology. These studies mainly employed methods that do not discriminate between 5mC and ShmC such that we do not know to what extent the reported association with stress comes from each individual modification. However, there is a growing literature looking specifically at ShmC in relation to stress but so far our understanding of the mechanisms by which stressors lead to changes in ShmC are less well-established than for 5mC. These studies primarily focus on rodent models of conditioned fear and extinction, which are considered to have significant face, construct, and predictive validity in relation to various fear-related disorders and aspects of exposure-based therapies [33].

ShmC in animals exposed to fear-related learning
Guo et al. [34] 2011 was the first study to demonstrate that neuronal activity regulates the expression of Tet enzymes and induces active DNA demethylation of critical plasticity-related genes such as Bdnf, which are known to be important in learning and memory formation. In addition, constitutive KO or deletion of Tet1 in mice results in reduced levels of global ShmC and decreased expression of several immediate early genes (IEGs) within both the cortex and hippocampus with altered IEG induction upon fear-related learning and memory processes [35]. In rodent models, cued and contextual fear conditioning have been used as ways to better understand pathological memory processes. Fear conditioning involves the repeated pairing of a neutral conditioned stimulus (CS, e.g. auditory cue or a particular environmental context) with an aversive unconditioned stimulus (US, e.g. footshock) following which the CS alone elicits autonomic and behavioural fear-conditioned responses, such as freezing. Extinction refers to the inhibition of these conditioned fear responses via repeated exposure to the CS alone, which in adult animals is due to new inhibitory learning rather than the erasure of the initial CS-US association [33]. Rudenko and colleagues showed that Tet1 KO mice display a lack of learning-induced active DNA demethylation around the transcription start site (TSS) of the IEGs Npas4 and Fos, which the authors suggest may contribute to the deficits observed in hippocampal synaptic plasticity associated with impairments in the extinction of contextual fear memory and spatial memory [35]. Kaas et al. [36] further demonstrated a role of Tet1 in the consolidation of cued-fear memory with viral-mediated overexpression of Tet1 within the mouse dorsal hippocampus leading to impairment in the retention of fear memory 24 h following conditioning. Tet1 overexpression also induced the upregulation of several IEGs and memory-associated transcripts within the hippocampus. However, similar fear memory impairments and hippocampal IEG induction was observed upon overexpression of a catalytically inactive Tet1 mutant. Together, these findings suggest that Tet1 may, to some degree, regulate basal activity or stimulus-evoked gene expression within the mouse brain independent of its DNA oxidation activity [36]. Such independent functions have been observed in mouse ESCs, where Tet1 associates with the Sin3a co-repressor complex to mediate transcriptional repression and Tet2 promotes histone modifications [37, 38]. A recent study also demonstrated that Tet1 as well as Tet1 mutants lacking either the catalytic or CXXC domains impair differentiation of a neuroblastoma cell line, thus confirming catalytically independent activity of Tet1 in this context [39]. Moreover, knockdown or deletion of Tet1 alters the expression of genes encoding the DNA methyltransferases (Dnmts) within mouse brain and ESCs, although this is partly dependent on DNA oxidation [40, 41]. Thus, further investigation is required to decipher the significance of catalytic and non-catalytic activity of Tet family enzymes in the context of neuronal function.

Although Tet1 has been most extensively studied within pre-clinical models, extinction of cued-fear memory in mice identified Tet3 to be regulated within the infralimbic region of the prefrontal cortex (ILPFC) [42]. The authors demonstrated that extinction induced an increase in Tet3 mRNA within the ILPFC 24 h following training with shifts in the genome-wide distribution of ShmC also observed within this brain region. Viral-mediated knockdown of Tet3 but not Tet1 expression within the PFC impaired the retention of extinction memory, indicative of the importance of dynamic regulation of Tet3 and ShmC in this mnemonic process. A recent study further demonstrated that Tet3 (and to some extent Tet1) dynamically regulates homeostatic synaptic plasticity, which is dependent on DNA oxidation as well as base excision repair pathways [43]. Collectively, these studies indicate that Tet enzymes and ShmC
play a critical role in the dynamic regulation of transcriptional states (both stable and poised) within post-mitotic neurons in response to neuronal activity and environmental stimuli, which appears to be important for the synaptic plasticity underlying the consolidation and maintenance of fear-related learning and memory processes. However, despite the evident role of Tet enzymes and ShmC in neuronal function and fear-related learning paradigms, our understanding of the regulation of DNA demethylation pathways in stress-related psychopathology remains equivocal.

**ShmC in animal models of stress exposure**

Several studies to date have investigated the global as well as gene specific response of ShmC profiles to multiple stressors in animals ranging from prenatal, adolescent, and adult developmental periods. Using locus specific TAB-seq analysis, Li et al. [44] showed that acute restraint stress (30 min) in late adolescent mice induces a global increase in ShmC levels in the hippocampus with a locus-specific increase of ShmC at a single CpG within the 3′UTR of the glucocorticoid receptor (GR) gene, Nr3c1. However, this study is limited by low sample sizes (n = 3–4) and a lack of Nr3c1 mRNA or protein expression to enable correlation of stress-induced changes in ShmC with GR expression in this context. A further study conducted by the same laboratory addressed these issues via investigation of genome-wide ShmC profiles and transcriptome regulation within the mouse hippocampus 1 h following a single 30 min acute restraint stress via chemical labeling-based ShmC profiling and RNA-seq. The authors demonstrated that acute restraint stress regulates ShmC profiles within the hippocampus with an enrichment of differentially hydroxymethylated regions within intragenic loci, yet only a small subset of these sites were associated with differentially expressed genes with known or potentially novel stress-related functions [45].

In non-human primates, we investigated genome-wide promoter methylation and hydroxymethylation profiles in the prefrontal cortices of rhesus macaques reared with their biological mother or in a maternal deprivation condition using methylated DNA immunoprecipitation analysis and ShmC enrichment, respectively, followed by microarray hybridization [46]. When examining global levels of 5mC and ShmC within promoter regions (~2000 to +400 bp from TSS), we found that the levels were highly correlated, and there was a positive correlation between gene expression and promoter ShmC in genes with intermediate/low CpG content or highly methylated promoters. Investigation of specific gene promoters revealed that several neurotransmitter receptor genes, including those encoding the dopamine D3 receptor (Drd3), α1 adrenergic receptor (Adra1a), and the α2 GABA receptor subunit (Gabra2), were found to be differentially hydroxymethylated with no changes in 5mC between the two groups of macaques. In another study of adolescent brains in a rat model of caregiver maltreatment, researchers examined global 5mC and ShmC levels in the hippocampus and amygdala using an antibody-based technique [9]. The authors found higher 5mC levels in the dorsal hippocampus of maltreated males compared to all other groups and in the ventral hippocampus as compared to females. Additionally, lower global ShmC levels in the amygdala (but not hippocampi) were found in males exposed to maltreatment as compared to those that were not. These studies provide preliminary evidence of dynamic regulation of ShmC within the hippocampus in response to various stress paradigms, yet further studies of different age groups, brain regions and stress models, particularly chronic stress, will be important in clarifying the role of ShmC and Tet enzymes in pre-clinical models of stress-related psychopathologies.

**Discussion**

In this review, we have examined the commonly used methods for detection of ShmC and described studies profiling ShmC in animals exposed to fear-related learning and acute stress paradigms throughout several development periods. Animal studies have demonstrated dynamic regulation of ShmC and Tet enzyme expression upon fear-related learning and memory, suggesting a potential role in pathological memory processes, such as those observed in stress-related disorders. Moreover, extinction is the underlying principle of exposure-based therapies for various stress-related psychiatric disorders such that the mal-adaptability of epigenetic states within post-mitotic neurons via Tet enzymes and ShmC may subserve the formation of inhibitory learning for successful suppression of pathological memory. Future studies in human patients will be critical to further investigate dysregulation of ShmC and Tet enzymes in such disorders. Pre-clinical studies have also revealed an effect of acute stress at various ages of development on ShmC in animal models, but additional studies will be needed to determine if these changes are biologically relevant. Moreover, ShmC levels should be profiled in human subjects exposed to stress and those who have developed fear-related disorders.

Several important limitations of the extant work must be recognized. First, different techniques were used for profiling ShmC in different studies such that the results are not always comparable. Additionally, global ShmC levels do not provide an accurate representation of the dynamic nature of ShmC at discrete genomic loci that we now know occurs in response to neuronal activity and various environmental stimuli. A further caveat is that 5mC and ShmC profiles significantly differ between neuronal and non-neuronal cells as well as between different neuronal subtypes within both the human and rodent brain [10, 47, 48]. Thus, it is critical that future studies focus on cell-type specific analysis of 5mC and ShmC in both human post-mortem brain and pre-clinical models of stress-related disorders. Development of low input methods for single-cell sequencing will also be crucial for the profiling of DNA modifications in specific subsets of cells within the brain (i.e. those activated upon a given stressor) [49]. Despite the limitations, the studies reviewed highlight the sensitivity of the hydroxymethylome to stress, providing a foundation to consider its potential role in the development of stress-related disorders.

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**References**

1. Penn N, Suwalski R, O’Riley C, et al. The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. *Biochem J* 1972;126:781–90.
2. Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 2009;324:929–30.

3. Brelling A, Lyko F. Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. Epigenet Chrom 2015;8:24.

4. Branco M, Ficz G, Reik W. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. Nat Rev Genet 2012;13:7–13.

5. Iurlaro M, Ficz G, Oxley D, et al. A screen for hydroxymethylcytosine and formylcytosine binding proteins suggests functions in transcription and chromatin regulation. Genome Biol 2013;14:R119.

6. Szulik M, Pallan P, Nocek B, et al. Differential Stabilities and Sequence-Dependent Base Pair Opening Dynamics of Watson–Crick Base Pairs with 5-Hydroxymethylcytosine, 5-Formylcytosine, or 5-Carb oxylysin e. Biochemistry 2015;54:1294–305.

7. Yardimci H, Zhang Y. Charting oxidized methylcytosines at base resolution. Nat Struct Mol Biol 2015;22:656–61.

8. Hahn M, Szabo P, Pfeifer G. 5-Hydroxymethylcytosine: a stable or transient DNA modification? Genomics 2014;104:314–23.

9. Doherty T, Forster A, Roth T. Global and gene-specific DNA methylation alterations in the adolescent amygdala and hippocampus in an animal model of caregiver maltreatment. Behav Brain Res 2016;288:55–61.

10. Lister R, Mukamel E, Nery J, et al. Global epigenomic reconfiguration during mammalian brain development. Science 2013;341:1237905.

11. Wagner M, Steinbacher J, Kraus T, et al. Age-Dependent Levels of 5-Methyl-, 5-Hydroxymethyl-, and 5-Formylcytosine in Human and Mouse Brain Tissues. Angew Chem Int Ed Engl 2015;54:12511–4.

12. Gross J, Pacis A, Chen G, et al. Tet1 is critical for neuronal DNA Hydroxymethylation, active DNA demethylation, and memory formation. Neuron 2013;79:1086–93.

13. Lunnon K, Hannon E, Smith R, et al. Variation in 5-hydroxymethylcytosine across human cortex and cerebellum. Genome Biol 2016;17:27.

14. Nestor C, Reddington J, Benson M, et al. Investigating 5-hydroxymethylcytosine (5hmC): the state of the art. Methods Mol Biol 2014;1094:243–58.

15. Thomson J, Nestor C, Meehan R. 5-Hydroxymethylcytosine Profiling in Human DNA. Methods Mol Biol. Totowa, NJ: Humana Press, 2015, 1–10.

16. Lu F, Liu Y, Jiang L, et al. Role of Tet proteins in enhancer activity and telomere elongation. Genes Dev 2014;28:2103–2119.

17. Dawlaty M, Brelling A, Le T, et al. Loss of Tet enzymes compromises proper differentiation of embryonic stem cells. Dev Cell 2014;29:102–11.

18. Sun Z, Terragni J, Borgaro J, et al. High-resolution enzymatic mapping of genomic 5-hydroxymethylcytosine in mouse embryonic stem cells. Cell Rep 2013;3:567–76.

19. Sun Z, Dai N, Borgaro J, et al. A sensitive approach to map genome-wide 5-hydroxymethylcytosine and 5-formylcytosine at single-base resolution. Mol Cell 2015;57:750–61.

20. Laird A, Thomson J, Harrison D, et al. 5-hydroxymethylcytosine profiling as an indicator of cellular state. Epigenomics 2013;5:655–69.

21. Booth M, Branco M, Ficz G, et al. Quantitative sequencing of 5-methylcytosine and 5-hydroxymethylcytosine at single-base resolution. Science 2012;336:934–7.

22. Yu M, Hon G, Szulwach K, et al. Tet-assisted bisulfite sequencing of 5-hydroxymethylcytosine. Nat Protoc 2012;7:2159–70.

23. Calabrese R, Valentini E, Ciccarone F, et al. TET2 gene expression and 5-hydroxymethylcytosine level in multiple sclerosis peripheral blood cells. Biochim Biophys Acta 2014;1842:1130–6.

24. Sherwani S, Khan H. Role of 5-hydroxymethylcytosine in neurodegeneration. Gene 2015;570:17–24.

25. van den Hove D, Chouliaras L, Rutten B. The role of 5-hydroxymethylcytosine in aging and Alzheimer’s disease: current status and prospects for future studies. Curr Alzheimer Res 2012;9:545–9.

26. Al-Mahdawi S, Virmouni S, Pook M. The emerging role of 5-hydroxymethylcytosine in neurodegenerative diseases. Front Neurosci 2014;8:397.

27. Zhubi A, Chen Y, Dong E, et al. Increased binding of MeCP2 to the GAD1 and RELN promoters may be mediated by an enrichment of 5-hmc in autism spectrum disorder (ASD) cerebellum. Transl Psychiatry 2014;4:e349.

28. Papale LA, Zhang Q, Li S, et al. Genome-wide disruption of 5-hydroxymethylcytosine in a mouse model of autism. Hum Mol Genet 2015;24:7121–31.

29. Dong E, Gavrin D, Chen Y, et al. Upregulation of TET1 and downregulation of APOBEC3A and APOBEC3G in the parietal cortex of psychotic patients. Transl Psychiatry 2012;2:e159.

30. Guidotti A, Dong E, Tueting P, et al. Modeling the molecular epigenetic profile of psychosis in prenatally stressed mice. Prog Mol Biol Transl Sci 2014;128:89–101.

31. Dong E, Dzitoyeva S, Matrisciano F, et al. Brain-derived neurotrophic factor epigenetic modifications associated with schizophrenia-like phenotype induced by prenatal stress in mice. Biol Psychiatry 2015;77:589–96.

32. Stankiewicz A, Swiergiel A, Lisowski P. Epigenetics of stress adaptations in the brain. Brain Res Bull 2013;98:76–92.

33. Samra JS, Guarneri E, Labenda G, et al. Tet1 Oxidase Regulates Tet-1-Dependent Epigenetic Gene Expression and 5-Hydroxymethylcytosine Level in the Brain. Mol Cell Biol 2013;33:3284–96.

34. Guo J, Ma D, Mo H, et al. Neuronal activity modifies the DNA methylation landscape in the adult brain. Nat Neurosci 2011;14:1345–51.

35. Rudenko A, Dawlaty M, Seo J, et al. Tet1 is critical for neuronal activity-regulated gene expression and memory extinction. Neuron 2013;79:1109–22.

36. Kaas G, Zhong C, Eason DE, et al. TET1 controls CNS 5-methylcytosine hydroxylation, active DNA demethylation, and neuronal differentiation. Nat Neurosci 2013;16:870–7.

37. Williams K, Christensen J, Pedersen M, et al. TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 2011;473:443–8.

38. Chen Q, Chen Y, Bian C, et al. TET2 promotes histone O-GlCNACylation during gene transcription. Nature 2013;493:561–4.

39. Gao J, Ma Y, Fu H, et al. Non-catalytic roles for TET1 protein negatively regulating neuronal differentiation through rsGAP3 in neuroblastoma cells. Protein Cell 2016;7:351–61.

40. Kumar D, Aggarwal M, Kaas G, et al. TET1 Oxidase Regulates Neuronal Gene Transcription, Active DNA Hydroxymethylation, Object Location Memory, and Threat Recognition Memory. Neuroepigenetics 2015;4:12–27.

41. Yang J, Guo R, Wang H, et al. Tet Enzymes Regulate Telomere Maintenance and Chromosomal Stability of Mouse ESCs. Cell Rep 2016;15:1809–21.
43. Yu H, Su Y, Shin J, et al. Tet3 regulates synaptic transmission and homeostatic plasticity via DNA oxidation and repair. Nat Neurosci 2015;18:836–43.
44. Li S, Papale LA, Kintner D, et al. Hippocampal increase of 5-hmC in the glucocorticoid receptor gene following acute stress. Behav Brain Res 2015;286:236–40.
45. Li S, Papale LA, Zhang Q, et al. Genome-wide alterations in hippocampal 5-hydroxymethylcytosine links plasticity genes to acute stress. Neurobiol Dis 2016;86:99–108.
46. Massart R, Suderman M, Provencal N, et al. Hydroxymethylation and DNA methylation profiles in the prefrontal cortex of the non-human primate rhesus macaque and the impact of maternal deprivation on hydroxymethylation. Neuroscience 2014;268:139–48.
47. Kozlenkov A, Wang M, Roussos P, et al. Substantial DNA methylation differences between two major neuronal subtypes in human brain. Nucleic Acids Res 2016;44:2593–612.
48. Mo A, Mukamel E, Davis F, et al. Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. Neuron 2015;86:1369–84.
49. Farlik M, Sheffield N, Nuzzo A, et al. Single-cell DNA methylome sequencing and bioinformatic inference of epigenomic cell-state dynamics. Cell Rep 2015;10:1386–97.