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TET enzymes and DNA hydroxymethylation in neural development and function — How critical are they?

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ABSTRACT

Epigenetic modifications of the genome play important roles in controlling gene transcription thus regulating several molecular and cellular processes. A novel epigenetic modification – 5-hydroxymethylcytosine (5hmC) – has been recently described and attracted a lot of attention due to its possible involvement in the active DNA demethylation mechanism. TET enzymes are dioxygenases capable of oxidizing the methyl group of 5-methylcytosines (5mC) and thus converting 5mC into 5hmC. Although most of the work on TET enzymes and 5hmC has been carried out in embryonic stem (ES) cells, the highest levels of 5hmC occur in the brain and in neurons, pointing to a role for this epigenetic modification in the control of neuronal differentiation, neural plasticity and brain functions. Here we review the most recent advances on the role of TET enzymes and DNA hydroxymethylation in neuronal differentiation and function.

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1. Introduction

DNA methylation is an epigenetic mark that has traditionally been regarded as a very stable modification of the genome. It occurs mainly at cytosines located on CpG dinucleotides although there is now evidence of significant amounts of cytosine methylation occurring in different contexts (mainly at CpA) in oocytes, embryonic stem cells and in neurons [46,47,63,84]. DNA methylation is known to be involved in several cellular and molecular mechanisms such as control of gene transcription, establishment of cellular identity, silencing of transposon elements, parental imprinting, X-chromosome inactivation and carcinogenesis [3]. Although the establishment and maintenance of DNA methylation patterns is now well-known, through the activity of the de novo DNA methyltransferases (DNMT3A, DNMT3B and DNMT3L) and preservation of this methylation using the maintenance methyltransferase DNMT1, the removal of methyl groups from the cytosines, i.e. demethylation, was less well understood until recently. Global epigenetic reprogramming (involving global loss of DNA methylation) occurs predominantly at two developmental stages — in the pronuclear zygote, after fertilization, and in primordial germ cells, during gonadal formation. There are mainly two ways of achieving DNA demethylation — through *passive* loss of methylation during DNA replication, in the absence of the enzyme that maintains methylation, DNMT1; and an *active* removal of methylation, that occurs without involvement of DNA replication. Although the first mechanism is better understood, for example by failure of DNMT1 to localize to replication foci [57],

the latter has been a subject of intense investigation to find the putative "DNA demethylase" process.

1.1. DNA methylation in the nervous system

The precise temporal regulation of de novo methylation and demethylation is of particular importance for the differentiation and maturation of the mammalian central nervous system (CNS) [55]. It has been shown that depletion of DNMT1 in post-mitotic neurons, despite its high transcription levels, did not affect global DNA methylation levels; on the other hand, DNMT1 deficiency in mitotic embryonic neural precursor cells (NPCs) resulted in DNA hypomethylation in daughter cells, with hypomethylated CNS neurons being functionally impaired and selected against in postnatal stages [12]. Dnmt1-deficient NPCs show precocious astroglial differentiation through demethylation of genes in the JAK-STAT pathway leading to an enhanced activation of STATs, which in turn triggers astrocyte differentiation [13]. Expression analysis of the de novo methyltransferases DNMT3A and DNMT3B revealed that whereas DNMT3B is only detected between embryonic days E10.5 and 13.5, when early neurogenesis occurs, DNMT3A is first detected at E10.5 through adulthood, being detected in NPCs, postmitotic CNS neurons and oligodendrocytes (being almost undetectable in GFAP-positive astrocytes). These results suggest that DNMT3B may be important for the early phase of neurogenesis whereas DNMT3A likely plays a dual role in regulating neurogenesis prenatally and CNS maturation and function postnatally [14]. Indeed, it was later shown that *Dnmt3a* is expressed in postnatal neural stem cells (NSCs) and is required for neurogenesis [80]. Although multiple isoforms of *Dnmt3a/3b* are present in mouse

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embryonic stem (ES) cells, only the *Dnmt3a* full-length variant is expressed in the known neurogenic niches in the postnatal brain – the subependymal (SEZ) and subventricular (SVZ) zones of the forebrain and the hippocampal dentate gyrus – where NSCs persist throughout life. Wu and colleagues have shown that *Dnmt3a* occupies and methylates intergenic regions and gene bodies flanking proximal promoters of a large cohort of transcriptionally permissive genes, many of which encode regulators of neurogenesis. Surprisingly, this non-promoter DNA methylation triggers the expression of these neurogenic genes by functionally antagonizing Polycomb repression, reducing H3K27me3 levels and PRC2 binding. Also, *Dnmt3a* represses glial differentiation genes. Together, these results suggest that the DNA methyltransferase *Dnmt3a* not only mediates repression in self-renewing postnatal NSCs by methylating proximal promoters, but also promotes transcription of targets, including neurogenic genes, by antagonizing Polycomb repression [80]. Additionally, Dnmt3a-deficient ES cells show a propensity to differentiate into astrocytes and oligodendrocytes [83]. *Dnmt3a* has also been implicated in regulating cellular and behavioral plasticity to emotional stimuli, by regulating DNA methylation in Nucleus Accumbens (NAc) neurons [42]. Moreover, *Dnmt1* and *Dnmt3a* double knockout (DKO) in forebrain excitatory neurons resulted in abnormal long-term plasticity in hippocampal CA1 region together with deficits in learning and memory. Although no neuronal loss was found, hippocampal neurons in DKO mice were smaller than wild-type and showed deregulated expression of genes involved in synaptic plasticity, possibly due to the observed decrease in DNA methylation [15].

Regarding the role of DNA methylation in neural plasticity, it has been shown that upon depolarization, post-mitotic neurons lose methylation at regulatory regions of the *Bdnf* gene, which correlates with higher transcript levels of this gene. This was proposed to occur through dissociation of the MeCP2-histone deacetylase-mSin3A repression complex from the promoter of *Bdnf* [52]. Methylation at the *Bdnf* gene has also been suggested to play a role in the consolidation of fear memory, since contextual fear conditioning resulted in increased *Bdnf* levels and decreased methylation at the exon IV promoter [50,89]. Moreover, methylation at the promoter of another gene, the Glucocorticoid Receptor (GR or NR3C1), has been shown to be modulated by maternal care and persisted through adulthood [76], suggesting that environmental cues can modulate the epigenetic state of some genes in post-mitotic cells such as mature neurons. Indeed, a more recent report has shown that around 3000 CpG sites were actively demethylated or de novo methylated in mouse dentate granule cells after synchronous neuronal activation. These sites were significantly enriched in low-CpG density regions associated with brain-specific genes related to neuronal plasticity [20]. Additionally, *Gadd45b*, a member of the Gadd45 family previously implicated in DNA repair, adaptive immune response [49, 71] and excision of DNA 5-methylcytosine in cultured cells [2], has been described to be essential for activity-dependent DNA demethylation and increased expression of the neurotrophic factors BDNF and FGF1 [51].

In NSCs differentiated from mouse ES cells, around 200 tissue-specific Differentially Methylated Regions (tDMRs) were identified and the genes enriched in these regions were involved in neural differentiation, including for example *Jag1* and *Tcf4* [7].

Recently, an interesting observation has been made regarding the role of DNA methylation in the myelination of Schwann cells. It has been shown that the myelination program is accompanied by a marked loss of DNA methylation at gene regulatory regions (promoters and putative enhancers) and repeat elements, that resulted in upregulation of myelination-associated genes such as genes involved in lipid metabolism. Additionally, the authors hypothesized that this decrease in DNA methylation could be due to significantly reduced levels of SAMe (S-adenosylmethionine, also known as AdoMet), which is the methyl group donor for cytosine methylation, in mature myelinating Schwann cells when compared to actively myelinating Schwann cells [72].

Interestingly, cytosine methylation in adult mouse and human neurons and frontal cortex is located not only at CpG dinucleotides but also at CpH sites (H = A/C/T), which represented around 25% of methylated sites in mouse dentate neurons and around 53% in human neuronal cells. Neuronal CpH methylation was enriched in regions of low CpG density, depleted at protein-DNA interaction sites and anti-correlated with gene expression [21,46]. Functionally, methylated CpH can repress transcription in vitro, is recognized by MeCP2 in vivo and is established during postnatal neuronal maturation and maintained by the de novo methyltransferase DNMT3A. Preferential loss of CpH methylation caused by DNMT3A knockdown was accompanied by the significant derepression of the mRNA expression of these CpH methylated genes [21].

Another interesting feature of the nervous system is the fact that more than 5% of the genes expressed in the brain display a degree of imprinted expression, i.e., monoallelic expression dependent on parental origin and regulated by Differentially Methylated Regions (DMRs). In fact, a number of neurological and psychiatric disorders have unexplained parent-of-origin effects that could be attributed to disruption of imprinted genes expressed in the brain [18,37].

2. DNA hydroxymethylation

In 2009, two seminal papers described the existence of another epigenetic modification, 5-hydroxymethylcytosine (5hmC) that was present in high levels in neurons and embryonic stem (ES) cells [40, 69]. In the mouse brain, 5hmC represented 0.6% of total nucleotides in Purkinje cells, which corresponded to as much as 40% the amount of 5-methylcytosine (5mC), and 0.2% of total nucleotides in granule cells [40]. In mouse ES cells, 5hmC represented around 0.04% of all nucleotides corresponding to 5 to 10% the levels of 5mC [69]. 5-hmC arises from the oxidation of 5-mC by a group of Fe^{2+} and 2-oxoglutarate-dependent dioxygenases belonging to the mammalian TET family, namely TET1, 2 and 3, which are homologs of the trypanosome JBP proteins that can oxidize the 5-methyl group of thymine. Tahiliani and colleagues were able to show that TET1 catalyzes the conversion of 5mC into 5hmC in vitro and that depletion of TET1 resulted in decreased levels of 5hmC in mouse ES cells [69]. These reports provided new insights into the mechanism of active DNA demethylation, suggesting that a hydroxylated methyl group could be an intermediate for oxidative demethylation or a stable modification that eliminates the need for removal of the methyl group by modulating the affinity of proteins that bind to methylated DNA or recruiting selective 5hmC binding proteins.

All TET proteins were later shown to be able to catalyze the conversion of 5mC into 5hmC and *Tet1* has been implicated in the maintenance of the pluripotent state in ES cells by regulating *Nanog* expression levels via promoter methylation. Furthermore, *Tet1* knockdown in preimplantation embryos resulted in a bias towards trophectoderm differentiation [33] as was also observed in *Tet1* knockdown in ES cells [16]. Additionally, *Tet1* knockdown downregulated a group of pluripotency-associated genes (for example *Esrrb*, *Klf2*, *Tcl1* and *Zfp42*) as a result of an increased methylation at their promoters [16].

In addition to converting 5mC into 5hmC, TET enzymes were shown to be capable of further oxidizing 5hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [26,34]. 5caC is specifically recognized and excised by TDG, suggesting a TDG-mediated base excision mechanism in active DNA demethylation [26]. Indeed, TDG depletion in mouse ES cells caused an accumulation of 5fC and 5caC at a large number of proximal and distal gene regulatory elements, in contrast to wild-type ES cells in which 5fC/5caC accumulates at major satellite repeats but not at nonrepetitive loci [62]. Altogether, these results point to a TET/TDG-dependent active DNA demethylation process.

Active DNA demethylation has been described to occur in the paternal genome soon after fertilization occurs [27]. Work by several

groups revealed that 5hmC accumulates in the paternal pronucleus at the beginning of S-phase (PN3), reaching a maximum in G2-phase (PN5), while 5mC concomitantly decreases [19,31,32,60,79]. *Tet3* is highly transcribed in oocytes and zygotes and localizes to the paternal pronucleus. Depletion of *Tet3* in the zygotes results in a failure to convert 5mC to 5hmC in the paternal pronucleus [19,79]. In addition to 5hmC, 5fC has also been shown to be present in the maternal and paternal genomes in early embryo development [73]. Genome-wide DNA demethylation also occurs in primordial germ cells (PGCs) resetting the epigenome prior to gametogenesis. 5hmC has also been shown to mediate this process in PGCs, driven by the high levels of *Tet1* and *Tet2*, and participate in the unique process of imprint erasure [23].

In ES cells, we and others have shown that 5hmC localizes at gene promoters and CpG-islands (CGIs), regions that are usually depleted of 5mC, and is associated with increased transcriptional levels [16]. Additionally, 5hmC was shown to be enriched in gene bodies of actively transcribed genes and in extended promoter regions of Polycomb-repressed developmental regulators [81]. 5hmC was particularly enriched at the start sites of "bivalent genes", which bear dual histone 3 lysine 27 trimethylation (H3K27me3) and histone 3 lysine 4 trimethylation (H3K4me3) marks and are usually found at developmentally-regulated genes in ES cells [58]. Indeed TET1 has been shown to bind to these bivalent domains (repressed genes) as well as H3K4me3-only promoters (actively-transcribed genes), mediating both expression of pluripotency-associated genes and repression of Polycomb-targeted developmental regulators [82].

Although Tet1-null ES cells show reduced levels of 5hmC and subtle changes in gene expression, they are pluripotent and support development of live-born mice in tetraploid complementation assay but display skewed differentiation toward trophectoderm in vitro. *Tet1* mutant mice are viable, fertile, and grossly normal, though some mutant mice have a slightly smaller body size at birth [10]. However, knockout of the paternal *Tet1* resulted in extensive dysregulation of imprinted genes in the offspring, leading to placental, fetal and postnatal growth defects resulting in early embryonic lethality. The authors observed aberrant hypermethylation of paternally expressed imprinted genes, both in PGCs and in sperm [86].

On the other hand, *Tet1* and *Tet2* double knockout (DKO) ES cells albeit pluripotent caused developmental defects in chimeric embryos and a fraction of the DKO embryos showed midgestation abnormalities with perinatal lethality (although viable and overtly normal mice were also obtained), maybe due to reduced 5hmC and increased 5mC levels and abnormal methylation at various imprinted genes [9]. Finally, *Tet1/2/3* triple knockout (TKO) embryonic stem cells were strongly depleted in 5hmC and impaired in their ability to differentiate into embryoid bodies and teratomas. Consistently, TKO ES cells contributed poorly to chimeric embryos and could not support embryonic development, possibly due to the observed promoter hypermethylation and deregulation of genes implicated in embryonic development and differentiation [8]. In addition, MEFs deficient in all three TETs could not be reprogrammed to induced pluripotent stem cells (iPSCs) due to a block in the mesenchymal-to-epithelial transition step, caused at least partly by defective activation of key miRNAs, which depend on oxidative demethylation promoted by TET and TDG [29].

An interesting observation was that ascorbic acid (AA), also known as Vitamin C, was shown to enhance the generation of 5hmC in vitro and in vivo, through direct interaction with the C-terminal catalytic domain of TET enzymes, which probably promotes their folding and/or recycling of the cofactor Fe^{2+} [54,87]. In mouse ES cells, AA significantly increases the levels of all 5mC oxidation products, particularly 5fC and 5caC, leading to a global loss of 5mC [87]. In fact, AA has been shown to regulate TET1 function in mouse ES cells, promoting DNA demethylation of many gene promoters and upregulation of demethylated germline genes [4], and in somatic cell reprogramming

[5]. Interestingly, high concentrations of AA are present in neurons which suggests a role in regulating TET enzymes and 5hmC levels in the context of neuronal function [65].

2.1. DNA hydroxymethylation in the brain

In line with the previous observations in mouse Purkinje and granule cells [40], 5hmC has been shown to be highly abundant in several brain regions, such as the hypothalamus, cerebral cortex, hippocampus, brainstem, olfactory bulb, spinal cord, cerebellum and retina. In these regions, the levels of 5hmC ranged from 0.4% to 0.7% per total cytosine, compared to levels below 0.2% in several other tissues such as the kidney, lung or liver [17,56]. Interestingly, the pituitary gland presented a much lower level of 5hmC (0.06%) supporting the hypothesis that high 5hmC content is related to neuronal function, rather than topography. Immunostaining of 5hmC revealed that the highest levels are detected in fully differentiated neurons of the dentate gyrus; interestingly, cells located in the subgranular zone (i.e. between dentate gyrus and hilus), where neural progenitors are located, showed reduced 5hmC levels [17]. However, NSCs derived in vitro from human ES cells also exhibited strong 5hmC staining that did not disappear or decline after 18 days of neuronal differentiation. In contrast, differentiation of progenitors towards oligodendrocyte lineages was characterized by progressive loss of 5hmC staining [60]. Using a more sensitive method for quantification of 5hmC, Szwagierczak and colleagues reported levels between 1.0 and 1.4% in the cerebellum, cortex or hippocampus, much higher than the reported levels of 0.3% for mouse ES cells [68]. Additionally, these authors showed that unlike ES cells in which *Tet1* is the most transcribed, *Tet3* was the most expressed in these three brain regions [68].

Genome-wide mapping of 5hmC in several mouse brain regions (cerebellum, hippocampus and frontal cortex) and neuronal cells showed an enrichment of 5hmC in intragenic regions (gene bodies) as well as in proximal upstream and downstream regions relative to the Transcription Start Sites (TSS) and was associated with more highly expressed genes, namely at developmentally activated genes. Additionally, 5hmC in the brain was shown to increase with age in specific gene bodies [46,53,64,67,74]. Szulwach and colleagues observed that genomic distributions of 5hmC in both hippocampus and cerebellum were distinct from those observed in ES cells, albeit with some overlapping features. 5hmC was enriched throughout gene bodies in the brain and it was also present in the bodies of active genes in ES cells (although to a lesser degree than that found in the brain). On the other hand, at the TSSs of repressed genes, 5hmC could co-occur with repressor complexes in ES cells, whereas 5hmC appeared to be largely depleted from TSSs in the brain. These data suggested distinct regulation of 5hmC in the brain as compared with ES cells, and may point toward diverse mechanisms regulating 5hmC at TSSs and gene bodies [67]. 5hmC in the brain was also shown to be abundant in synaptic genes and to mark exon–intron boundary, whereas this boundary change was mainly due to 5mC in non-neural contexts [38,77]. Furthermore, at distal regulatory elements, 5hmC was shown to be negatively correlated with H3K27me3- and H3K9me3-marked repressive genomic regions and positively correlated with H3K4me2 and H3K27ac, and to be more enriched at poised enhancers than active enhancers in the brain and neural tissues, suggesting that hydroxymethylation might be an early event in enhancer activation [61,77]. In the human brain, genomic mapping of 5hmC correlated with the observations in the mouse, showing 5hmC enrichment at promoters and gene bodies, positively correlated with gene expression levels [35,74,77]. 5caC and 5fC have also been detected in human and mouse brain albeit at much lower levels than 5hmC [48].

Concerning DNA hydroxymethylation and neural plasticity, *Tet1* and *Apobec1* were shown to be involved in neuronal activity-induced, region-specific, active DNA demethylation and subsequent gene expression in the dentate gyrus of the adult mouse brain in vivo [22]. The

authors observed that overexpression of *Tet1* in the adult mouse dentate gyrus further increased 5hmC levels; on the other hand, overexpression of *Aid* (*Aicda*) significantly decreased the endogenous levels of 5hmC, supporting a role for 5hmC removal in vivo. Overexpression of both *Tet1* and *Aid* led to a decrease in the levels of DNA methylation in the promoters of two genes previously shown to exhibit neuronal activity-induced active DNA demethylation in the adult dentate granule cells, *Bdnf IX* and *Fgf1B* [51]. On the other hand, *Tet1* and *Apobec1* knockdown reduced ECS-induced demethylation of both *Bdnf IX* and *Fgf1B* promoters in the dentate neurons, preventing their activation [22].

MeCP2 (methyl-CpG-binding protein 2) was identified as the major 5hmC-binding protein in the brain [53]. Additionally, other 5hmC readers have been described in ES cells, NPCs and in the brain, with *Wdr76*, *Thy28(Thyn1)* and *Neil1* overlapping between the three cell types [66], suggesting that 5hmC might be not only a demethylation intermediate but also an epigenetic regulator.

2.2. DNA hydroxymethylation during neural development and neurogenesis

The development of mammalian brain is a spatial temporally orchestrated process that requires appropriate gene regulation to allow the NSCs to differentiate into distinct cell types such as neurons and glia (astrocytes and oligodendrocytes) [25]. Consequently, control of DNA methylation and hydroxymethylation is required for proper neural cell differentiation (Fig. 1; our unpublished observations).

As mentioned before, *Tet1* has also been implicated in neural progenitor cell proliferation in adult mouse brain, since mice lacking *Tet1* exhibited impaired hippocampal neurogenesis which affected cognitive brain functions such as learning and memory [88].

Hahn and colleagues investigated patterns of 5mC and 5hmC during neurogenesis in the embryonic mouse brain. They have shown that 5hmC levels increase during neuronal differentiation and are enriched at enhancers but associates preferentially with gene bodies of activated neuronal function-related genes. Within these genes, gain of 5hmC is often accompanied by loss of H3K27me3. Enrichment of 5hmC was not associated with substantial DNA demethylation, suggesting that 5hmC is a stable epigenetic mark (see review by Pfeifer and colleagues, this issue). Functional perturbation of the H3K27 methyltransferase *Ezh2* or of *Tet2* and *Tet3* led to defects in neuronal differentiation, suggesting that the formation of 5hmC and loss of H3K27me3 cooperate to promote brain development [24].

Tet3 has been reported to play an essential role in early eye and neural development in *Xenopus* by directly regulating a set of key developmental genes. This targeting was critically dependent on the CXXC domain which was able to bind to unmodified cytosines followed by A, T, C or G with a slight preference for CpG dinucleotides. *Tet3* was shown to not only control the dynamics of 5mC/5hmC at target gene promoters but also was required for target gene regulation and biological function in *Xenopus* early embryonic development [85]. Another recent report shed light on the role of *Tet3* in neural differentiation. Li and colleagues took advantage of the recent TALEN-mediated gene targeting strategy to generate *Tet3* knockout ES cells. They were able to show that Tet3-null ES cells appear normal in self-renewal and maintenance but are impaired in neuronal differentiation. They observed that NPCs could be induced efficiently from *Tet3* knockout ES cells but undergo apoptosis rapidly and the terminal differentiation of neurons is greatly reduced [43].

During mature olfactory sensory neuron (mOSN) development, 5hmC has been shown to increase over gene bodies with substantial patterning occurring between the progenitor and mOSN stages. Although gene-body 5hmC levels correlated with gene expression in all developmental cell types – multipotent stem cells, neuronal progenitors and mOSN – this association was particularly pronounced within mOSNs [6]. Overexpression of *Tet3* in mOSNs markedly altered gene-body 5hmC levels and gene expression in a manner consistent with a positive role for 5hmC in transcription. Moreover, *Tet3* overexpression disrupted olfactory receptor expression and the targeting of axons to the olfactory bulb, key molecular and anatomical features of the olfactory system. These results suggested a physiological significant role for gene-body 5hmC in transcriptional facilitation and the maintenance of cellular identity independent of its function as an intermediate to demethylation [6].

A recent report has shown that TET proteins can be regulated by calpains at the postranscriptional level, during differentiation of mouse ES cells into NPCs [75]. Specifically, the authors have shown that all three TET enzymes are direct substrates of calpains and that calpain1 mediates TET1 and TET2 turnover in mouse ES cells and calpain2 regulates TET3 levels during neural differentiation.

As observed for DNA methylation [7], differentially hydroxymethylated regions (dHMRs) were also observed following in vitro differentiation of ES cells into NPCs [70]. The authors observed a reduction in 5hmC levels from ES cells to NPCs that affected mostly exons and
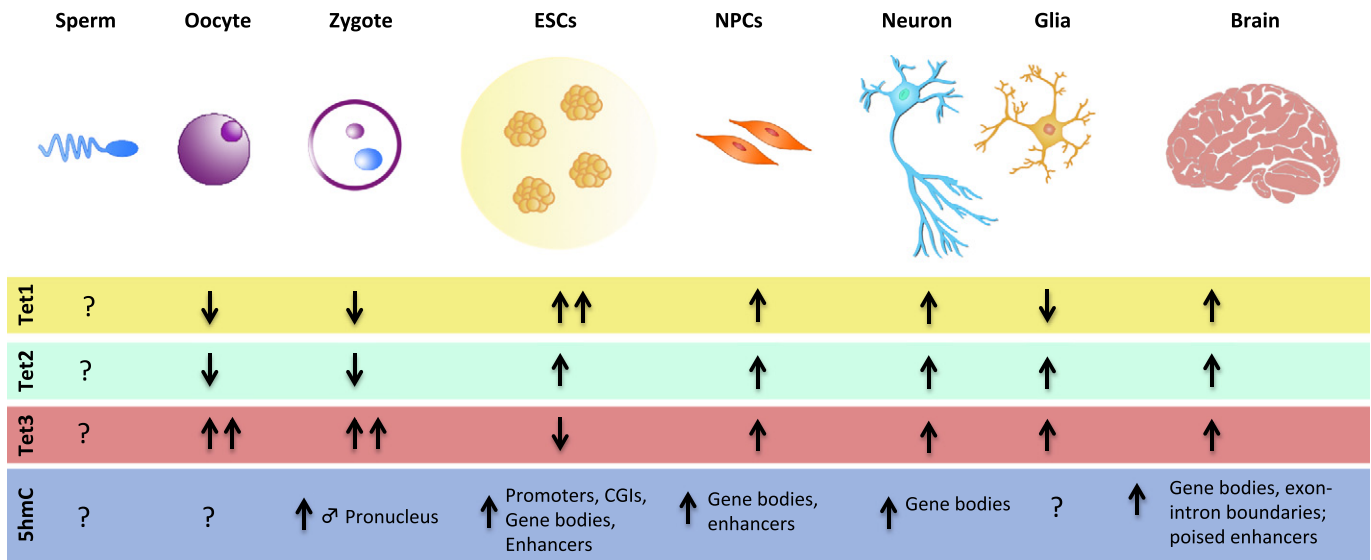

	Sperm	Oocyte	Zygote	ESCs	NPCs	Neuron	Glia	Brain
Tet1	?	↓	↓	↑↑	↑	↑	↓	↑
Tet2	?	↓	↓	↑	↑	↑	↑	↑
Tet3	?	↑↑	↑↑	↓	↑	↑	↑	↑
5hmC	?	?	↑ ♂ Pronucleus	↑ Promoters, CGIs, Gene bodies, Enhancers	↑ Gene bodies, enhancers	↑ Gene bodies	?	↑ Gene bodies, exon-intron boundaries; poised enhancers

Fig. 1. Schematic representation of the levels of TET enzymes and 5hmC enriched regions in several developmental cell stages. Arrow(s) up — increased expression/levels; Arrow down — decreased expression/levels.

promoters. However, some regions gained 5hmC and were associated with dendrite morphogenesis and other neural system functions. Unlike in ES cells, the authors did not find a positive correlation between 5hmC peaks in gene bodies and gene expression levels [70].

Regarding the other oxidative derivatives, it has been recently shown that 5caC transiently accumulates in the embryonic brain between 11.5 and 13.5 dpc and during neuronal and glial differentiation of NSCs. Additionally, the authors implicate TDG in the excision of both 5caC and 5fC during NSCs differentiation, since TDG knockdown led to increased levels in glial differentiation [78].

2.3. TET enzymes and DNA hydroxymethylation regulate brain functions

So far, the studies concerning the function of TET proteins in the brain are mostly focusing on TET1 [36,59,88]. In fact, both *Tet1* and *Tet2*-deficient mice survive, although the latter were observed to develop myeloid malignancies [10,39,45]. On the other hand, the combined knockdown of both genes results in perinatal lethality, albeit viable and overtly normal mice have also been obtained [9]. Also, deletion of *Tet3* leads to neonatal lethality [19] (Table 1).

Regarding the effects of *Tet1* ablation in brain morphology, no obvious alterations were found [59,88], since the number of neurons in different brain regions, as well as brain weight are maintained [59]. However, although *Tet1* deficiency is compatible with brain development, a significant decrease in 5hmC levels was found [59]. Concerning the effects of *Tet1* deficiency upon behavior of adult mice, some interesting alterations were described by Zhang and collaborators. Tet1 knockout mice presented a delay in learning and significant deficiency in short-term memory retention, indicating a cognitive disorder. These alterations in memory might be explained through the functional role of *Tet1* in adult neurogenesis. Nestin-GFP transgenic mice crossed with *Tet1* knockout mice were used to follow NPCs. The results revealed a strong decrease in GFP-positive cells, suggesting that *Tet1* is a key regulator for the neural progenitor cell pool in the hippocampal dentate gyrus. Consistently, a great enrichment of a cohort of genes containing high promoter hypermethylation was found [88]. Several of these genes are involved in neural progenitor proliferation, neuroprotection, and mitochondria function, including *Galanin(Gal)*, *Ng2(Cspg4)*, *Ngb*, *Kctd14*, and *Atp5h*. Particularly, *Galanin* and *Ng2* are known as critical players on neurogenesis regulation [1,11,41]. In summary, these results demonstrate that adult neurogenesis may be regulated, in a significant part, by *Tet1* through DNA demethylation [88].

In addition, ablation of *Tet1* in mice revealed impairments in memory extinction [59], a form of inhibitory learning, which allows an adaptive control of cognition and reveals mental plasticity [28]. While the control mice overcame their fear when the contextual danger was removed, the knockout group failed to display any memory extinction. Synaptic plasticity seems also to be affected. Although normal basal synaptic transmission and long-term potentiation (LTP) remain unaffected, long-term depression (LTD) was abnormally enhanced in *Tet1* KO mice. This fact may be possibly triggered by downregulation of plasticity-related neuronal genes (*Npas4*, *Fos*, *Arc*) in both the hippocampus and cortex. Interestingly, the analysis of the key regulatory gene, *Npas4*, has shown hypermethylation on the promoter region. This epigenetic modification may be responsible for the deregulation of its target genes, impairing cognitive processes [59].

On the other hand, Kaas and colleagues established a viral-mediated approach to overexpress *Tet1* or its mutated version (catalytically inactivated) in adult hippocampus and studied its involvement in memory formation. Overexpression of *Tet1* catalytic domain leads to an increase in 5mC to 5hmC conversion, as well as transcription levels of genes involved in the TET-mediated DNA demethylation pathway. Some of these genes are involved in synaptic plasticity and memory formation. However, the upregulation of memory-related genes such as *Arc*, *Fos* and *Homer1* did not seem to be dependent on the 5mC to 5hmC conversion (at least in the hippocampal region CA1), since the expression levels of these genes were not altered by the catalytic inactivation of *Tet1*. This suggests a 5mC oxidation-independent role of *Tet1* in adult brains. In the absence of its 5mC substrate, *Tet1* retains the capacity to regulate the expression of its target genes, possibly through an allosteric mechanism. On the other hand, overexpression of memory-associated genes led to an impairment of contextual fear memory, which may be explained by the increase of *Fos* and *Egr2* expression and subsequent activation of their downstream targets [36].

Little is known about the role of both *Tet2* and *Tet3* in neuronal differentiation and function, apart from a study showing that neuronal differentiation is accompanied by an up regulation of these proteins in parallel with the increase of 5hmC levels [24]. In addition to the prevalent demethylation activity of *Tet1* in adult hippocampus [22], *Tet3* is highly expressed in the cortex of adult mice [68] and higher levels of 5hmC were found in the cortical neurons of adult brain [40]. Furthermore, *Tet3* knockdown via intra-Infralimbic-Prefrontalcortex (ILPFC) shRNA leads to a significant impairment in fear extinction memory. A particular gene, *Gephyrin(Gphn)*, directly involved in fear extinction was studied. The increase of *Gephyrin* mRNA was transient 2 h after extinction training, returning to the baseline 24 h after. Also *Tet3* occupancy was transiently surrounding the *Gephyrin* gene, suggesting that DNA methylation can be continually altered after learning. This fact reveals that *Tet3* activity within

Table 1
Phenotypes of TET enzymes knockout (KO) and knockdown (Kd) in ES cells, NPCs and in the brain.

Enzyme	KO/Kd	Cell/tissue type	Phenotype	References
TET1	KO	ES cells	Remain pluripotent but skewed differentiation towards trophectoderm	[10]
		Brain	Delay in learning capacity; impairment in short-term memory; decrease in NPCs population in hippocampal DG; impairment in memory extinction; Long-term depression (LTD)	[10,59,88]
	Kd	ES cells	Bias towards trophectoderm differentiation	[16]
		Brain	Failed to activate neuronal activity-induced genes	[22]
TET2	KO	Brain	Mice are viable; So far no studies in the brain	[39,45]
	Kd	ES cells	Decrease of 5hmC in gene bodies and boundaries of exons	[30]
		NPCs	Defects in neuronal differentiation	[24]
TET3	KO	ES cells	Impaired in neuronal differentiation	[43]
		NPCs	Impaired terminal differentiation of neurons	[43]
		Brain	Neonatal lethality	[19]
	Kd	ES cells	Failure to convert 5mC to 5hmC in the paternal pronucleus	[79]
		Brain	Impairment in fear extinction	[44]
TET 1/2	Double Knockout	ES cells	Pluripotent cells but caused developmental defects; embryos showed midgestation abnormalities with perinatal lethality;	[9]
TET 1/2/3	Triple Knockout	ES cells	Impaired ability to differentiate into embryoid bodies and teratomas; could not support embryonic development	[8]

ILPFC is a key regulator for the learning-dependent accumulation of 5hmC conditioning rapid behavioral adaptation [44].

In spite of all the advances, future studies addressing the impact of TET depletion should be a key contribution to better understand how the absence of these enzymes influence brain function. The elucidation of how TET enzymes and hydroxymethylation are regulated may provide potential advances in treatments for brain disorders.

3. Concluding remarks

Recent progress in the elucidation of the role of TET enzymes and hydroxymethylation has significantly contributed to our understanding of the process of DNA demethylation. In addition, 5-hydroxymethylcytosine seems to have a role on its own in regulating gene expression and cellular processes (see also reviews by Hajkova and colleagues, as well as Wen and Tang, this issue). Particularly in the nervous system, TET enzymes and 5hmC are present at high levels and recent studies have highlighted their critical role in regulating both neural differentiation and brain function. Despite all the advances in the understanding of 5hmC and its derivatives synthesis, genomic distribution and function, much remains to be unraveled. Specifically in the topic of this review, it remains to be determined what are the effects of TET and 5hmC depletion in specific brain regions and neuronal cell types and neural precursor cells fate determination. Given the pace that new discoveries are being made, we are sure to see novel and exciting findings in the near future.

Acknowledgments

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