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Demethylation in Early Embryonic Development and Memory

Carol Bernstein and Harris Bernstein

Abstract

DNA repair processes arose early in evolution. During evolution, DNA base excision repair apparently acquired additional roles in demethylation of cytosines in DNA. Demethylation is central to two mammalian fundamental processes. Embryonic reprogramming and neuronal memory require rapid gene expression alterations depending in part on demethylations. The active demethylation reactions in both processes primarily depend, first, on the family of 5-methylcytosine oxidases sharing the acronym ten-eleven translocation (TET methylcytosine dioxygenases) and, second, on DNA base excision repair enzymes. In mice, within 6 h of fertilization, the paternal chromosomes are close to 100% actively demethylated through TET and repair activity. (Methylation of maternal DNA is blocked during subsequent cycles of replication, so methyl groups on maternal DNA, passively, becomes highly diluted over the next 4 days.) Rats subjected to one instance of contextual fear conditioning create an especially strong long-term memory. At 24 h after training, 9.2% of the genes in the rat genomes of hippocampus neurons are differentially methylated, including over 500 genes with demethylation. The emergence of embryonic development in evolution depended on preexisting DNA methylation/demethylation pathways to modify gene expression. The further emergence of memory likely evolved from the earlier set of methylation/demethylation capabilities associated with embryonic development.

Keywords: TET enzymes, OGG1, epigenetic, base excision repair, DNA repair, neuroepigenetics, neurogenesis, brain evolution

1. Introduction

DNA repair processes have a central role in epigenetic demethylation reactions that are employed in both early embryonic development and in memory. DNA likely emerged as the genetic material as long as 3.5 billion years ago [1]. From its inception as the genetic material, DNA was likely subject to damage. In present day organisms damage to DNA is frequent and occurs due to both metabolic and hydrolytic processes [2] as well as a result of environmental agents such as UV light and ionizing radiation. Thus, enzymes promoting DNA repair likely have been retained based on their adaptive benefit since early evolution. Currently, in humans, about 169 different DNA repair proteins have been identified [3]. During the course of evolution, many of these DNA repair proteins developed more than one enzymatic capability. For instance, at least 17 DNA repair proteins act in both a DNA repair pathway and in an apoptosis pathway [4]. These dual role proteins are required

for DNA repair when DNA damages are at relatively low levels but are active and required for apoptosis when DNA damages are at high levels.

In addition to the multiple roles of some DNA repair proteins, some endogenously produced DNA damaging agents also appear to have multiple roles. Reactive oxygen species (ROS) are produced by mitochondria during oxidative metabolism, and a small proportion are released from the mitochondria and interact with proteins, lipids and DNA to alter their structures. ROS can damage DNA in ways that are mutagenic or disruptive to expression. Thus, excessive ROS can cause mutations and other alterations leading to cancer [5]. However, ROS can interact with DNA to serve important positive roles. A large body of literature has shown the necessary roles of appropriate levels of ROS in embryonic development [6, 7] and in learning and memory [8, 9].

2. Demethylation in embryogenesis

During early embryogenesis of mammals, pathways of rapid demethylation are employed at multiple DNA sites to form totipotent cells. Subsequently, locally deposited methylations enable formation of subsets of cells that became specialized tissue types, such as primordial germ cells and neuronal stem cells [10]. Such rapid demethylations and subsequent methylations have also now been found to occur in the formation of memories and learning [11] and in both cases the mechanism of methyl group removal occurs by similar pathways involving TET enzymes and base excision DNA repair.

In embryogenesis, rapid and large scale demethylations occur at two stages [12]. One extensive set of demethylations occurs within a few hours after the sperm enters the egg, forming the zygote. Almost all methyl groups are removed from the paternal-origin chromosomes within 6 h of forming the zygote, before any replication has occurred [13]. Another extensive demethylation occurs early in embryogenesis, in the nuclei of the primordial germ cells shortly after they devolve from the other cells which are forming somatic tissues [14]. This stage of demethylation occurs in two phases. There is a first phase of rapid proliferation without methylation, causing dilution of methylation with a loss of methylation at almost all genomic sequences. Then there is a second phase, involving specific sites including germ-line and meiosis specific genes, where the demethylation is active and proceeds by pathways involving TET enzymes and base excision DNA repair.

Methylation of sites (which can be demethylated) in mammalian DNA are usually restricted to cytosines, forming 5-methylcytosine (5mC) (**Figure 1**). In this figure, the addition of a methyl group at the 5 position of cytosine is shown within a red oval. Of all the cytosines in DNA, the 5mCs occur primarily at “CpG” sites [16]. A CpG site is where a cytosine in a DNA strand is followed by a guanine nucleotide in the linear sequence of bases along the 5' to 3' direction. There are 28 million CpG sites in the human genome [17]. In humans, about 60% of the 28 million CpG sites are methylated in most somatic tissues [18]. CG dinucleotides (CpG sites) represent about 1% of total bases in the mammalian genome [19]. Three DNA methyltransferases in humans can methylate a base in DNA. These enzymes show a strong preference for methylating cytosines in CpG sites [20].

Mouse DNA is very similar to human DNA, with about 99% of mouse genes having a homolog in the human genome, and mice and humans having about the same number of genes [21]. However, the mouse sequence is about 14% shorter than the human sequence [21]. The mature mouse sperm genome has 80–90% overall methylation of its CpG sites, the highest global DNA methylation level of

any cell in the mouse [12]. Because of its shorter sequence, we can speculate that there may be fewer than 28 million CpG sites in the mouse genome, perhaps 86% as many as in the human genome, or about 24 million CpG sites. Thus, of the likely 24 million CpG sites, there are about 19–22 million methylated sites in mouse sperm DNA. In mouse zygotes, partial demethylation of the paternal nucleus is already evident 3 h after formation of the zygote [13]. By 6 h, demethylation of the paternal nucleus appears to be complete (**Figure 2**). During the subsequent first mitosis, there is just a small but significant residual methylation signal in some but not all of the paternally derived chromosomes [13]. By 3–4 days after fertilization, after replication to generate 16 cells, the embryo has formed a morula (a round body of cells with no differentiation) (**Figure 2**). By this time both the paternal and maternal chromosomes have mixed together in a single nuclear area and all have very low levels of methylation (In **Figure 2**, the methylation levels of the paternal and maternal chromosome are approximately represented by the blue lines during the period they can be distinguished. When the chromosomes become mixed, after two mitoses, the methylation level of the mixed chromosomes is represented by a brown line).

The almost complete demethylation of the zygote DNA in the paternal chromosomes at 22–25 million CpG sites occurs before any DNA replication. Thus, it occurs by an active process not connected to replication. The demethylation of the maternal chromosomes appears to largely take place by blockage of the methylating enzymes from acting on maternal-origin DNA and dilution of the methylated

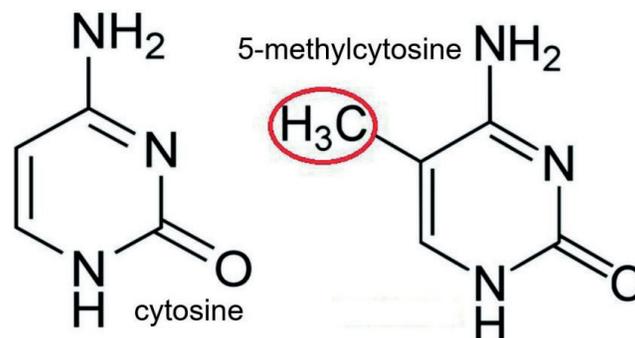


Figure 1. DNA methylation most often is the addition of a methyl group to cytosine in DNA. The image shows cytosine and 5-methylcytosine. In mammals, DNA methylation most frequently occurs at a cytosine followed by guanine in the DNA [15].

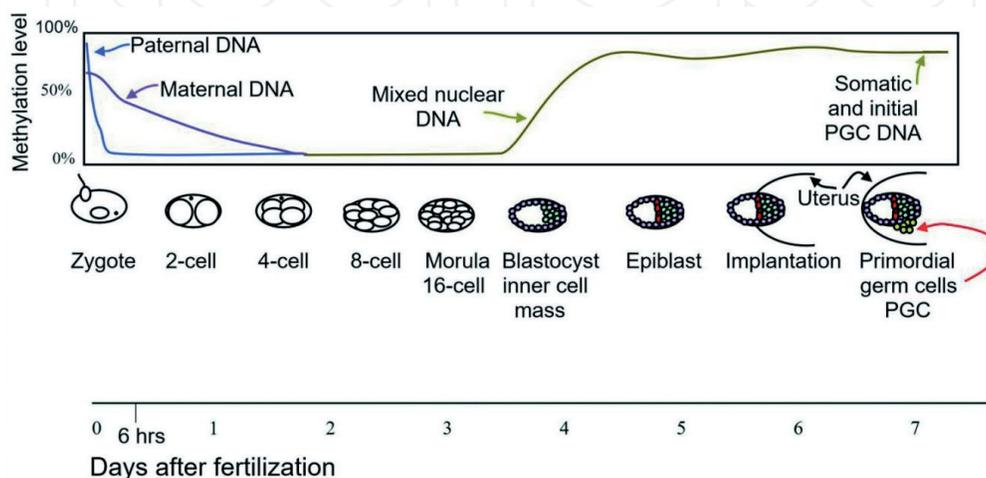


Figure 2. Methylation levels during mouse early embryonic development.

maternal DNA during replication. At the second metaphase after fertilization, maternal chromosomes showed methylation on only one of the two sister chromatids. This sister chromatid differentiation is consistent with mostly replication-dependent passive maternal chromosome demethylation [22]. Consequently, four-cell embryos have a much lower methylation density over the maternal nuclear compartment. Methylation of the maternal genome further decreases with every additional replication cycle. The morula (at the 16 cell stage), overall, has much reduced methylation of DNA.

High levels of *de novo* DNA methylation then occur in the cells of the inner cell mass of the blastocyst, to establish the specific methylation patterns of principal cell lineages in the early embryo [13]. Afterwards, by day 5 of mouse embryogenesis, the epiblast is formed, followed by implantation of the epiblast in the uterine epithelium (**Figure 2**). By day seven after fertilization, the newly formed primordial germ cells (PGC) in the implanted embryo devolve from the remaining somatic cells. At this point the PGCs have high levels of methylation. These cells migrate from the epiblast along the hindgut toward the genital ridges starting about day 7.8. By day 8.5 they are rapidly proliferating and beginning demethylation in two waves. In the first wave, demethylation is by replicative dilution, but in the second wave demethylation is by an active process. The second wave, during days 9.5–13.5, leads to demethylation of specific loci. At day 13.5, the PGC genomes display the lowest levels of DNA methylation of any cells of the mouse in the entire life cycle [14].

2.1 Mechanisms of demethylation

The demethylation of methylated CpG sites of DNA occurs in three stages: (1) recruitment of a TET enzyme to initiate demethylation (although there is one minor mechanism that does not utilize a TET enzyme); (2) intermediate steps of oxidation or oxidative deamination (forming intermediate products of demethylation); and (3) culminating steps of DNA base excision repair resulting in final replacement of 5-methylcytosine with cytosine.

The pathways by which demethylation can occur [23] are shown in outline in **Figure 3**. This figure indicates two types of oxidation reactions that may occur in demethylation. One occurs by oxidation of the added methyl group at the 5 position of cytosine. The other occurs through oxidative deamination of the amine group at the 4 position of cytosine. The pathway on the left depends on oxidation of each of the adducts on the 5 position of cytosine, sequentially, by a TET enzyme, followed by action of base excision repair (BER) enzymes. TET enzymes (ten-eleven translocation methylcytosine dioxygenases) oxidize adducts on cytosine in an iron and alpha-ketoglutarate dependent process. This TET-type dependent pathway likely carries out the bulk of the demethylations discussed here. However, as reviewed [25], two other pathways involving AID/APOBEC and base excision repair enzymes can occur. In one pathway there is an initial TET reaction. The other pathway involving AID/APOBEC results in oxidative deamination of 5mC directly to thymine followed by base excision repair. The activity of AID/APOBEC appears to cooperate with a TET enzyme in neuronal functions [26]. It is notable that demethylation, in all its pathways, employs the enzymes of the base excision repair pathway.

3. Base excision repair

In **Figure 3**, base excision repair is indicated by the highlighted acronym “BER”. To complete the description of the mechanism shown above, we include a

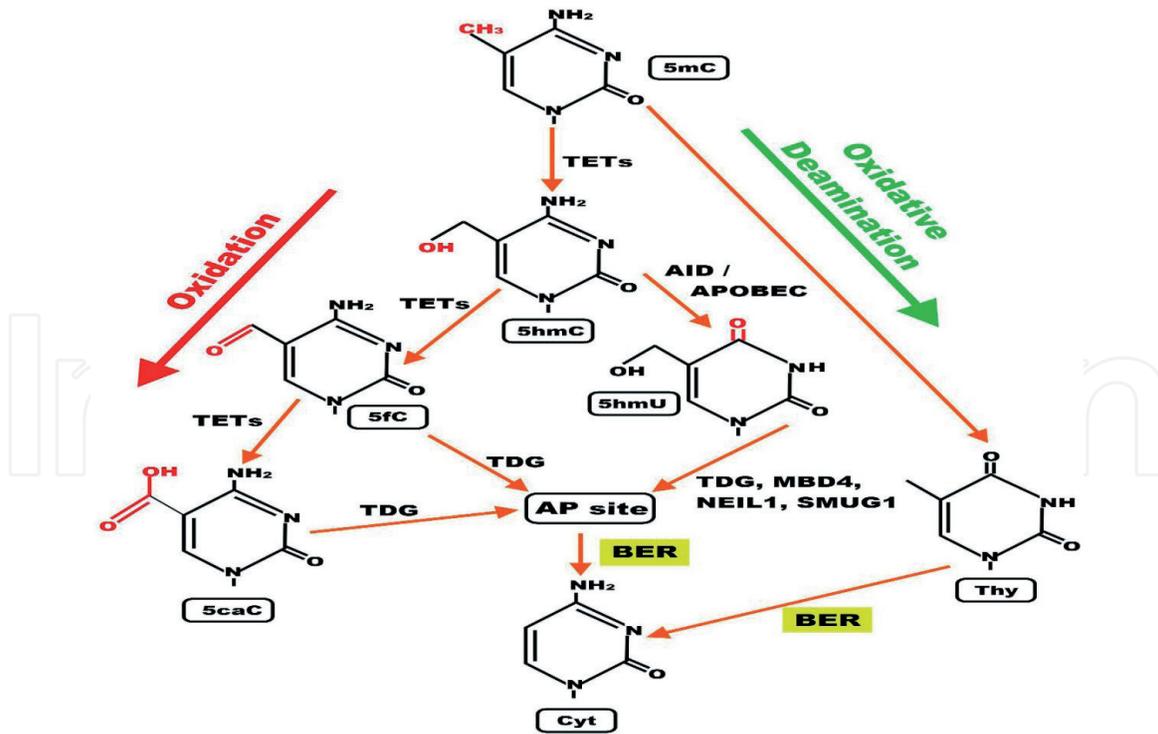


Figure 3. Demethylation of 5-Methylcytosine (5mC) in neuron DNA. As reviewed in [23], in brain neurons 5mC is oxidized by the ten-eleven translocation (TET) family of dioxygenases (TET1, TET2, TET3) to generate 5-hydroxymethylcytosine (5hmC). In successive steps TET enzymes further hydroxylate 5hmC to generate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Thymine-DNA glycosylase (TDG) recognizes the intermediate bases 5fC and 5caC and excises the glycosidic bond resulting in an apyrimidinic site (AP site). In an alternative oxidative deamination pathway, 5hmC can be oxidatively deaminated by activity-induced cytidine deaminase/apolipoprotein B mRNA editing complex (AID/APOBEC) deaminases to form 5-hydroxymethyluracil (5hmU) or 5mC can be converted to thymine (Thy). 5hmU can be cleaved by TDG, single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), Nei-Like DNA Glycosylase 1 (NEIL1), or methyl-CpG binding protein 4 (MBD4). AP sites and T:G mismatches are then repaired by base excision repair (BER) enzymes to yield cytosine (Cyt). Figure by [24].

diagram illustrating the base excision repair pathway used in the latter stages of the conversion of 5mC to C (**Figure 4**). In this diagram the two strands of DNA are represented by parallel horizontal lines. With the first downward arrow we show thymine DNA glycosylase (TDG) removing 5-formylcytosine (5fC) from the DNA backbone, leaving an *apyrimidinic site*. Then AP endonuclease cleaves the 5' deoxyribose-phosphate in the DNA backbone of a single strand, leaving a 3' hydroxy end and a 5' deoxyribose phosphate end (second downward arrow). This is followed by either short patch or long patch repair. In short patch repair, 5' dRP lyase trims the 5' dRP end to form a phosphorylated 5' end. This is followed by DNA polymerase β adding a single cytosine to pair with the pre-existing guanine in the complementary strand and then DNA ligase to seal the cut strand. In long patch repair, DNA synthesis is thought to be mediated by polymerase δ and polymerase ϵ performing displacing synthesis to form a flap. Pol β can also perform long-patch displacing synthesis. Long-patch synthesis typically inserts 2–10 new nucleotides. Then flap endonuclease removes the flap, and this is followed by DNA ligase to seal the strand.

In an example below (see “Targeting TET to 5-methylcytosine”) we show that, in at least one well documented case, the ROS-induced damage of 8-OHdG at a CpG site initiates demethylation. In the base excision pathways shown in **Figure 4**, it is not clear at what stage 8-OHdG itself may be removed. Thus, 8-OHdG is allowed to remain in most steps of this diagram.

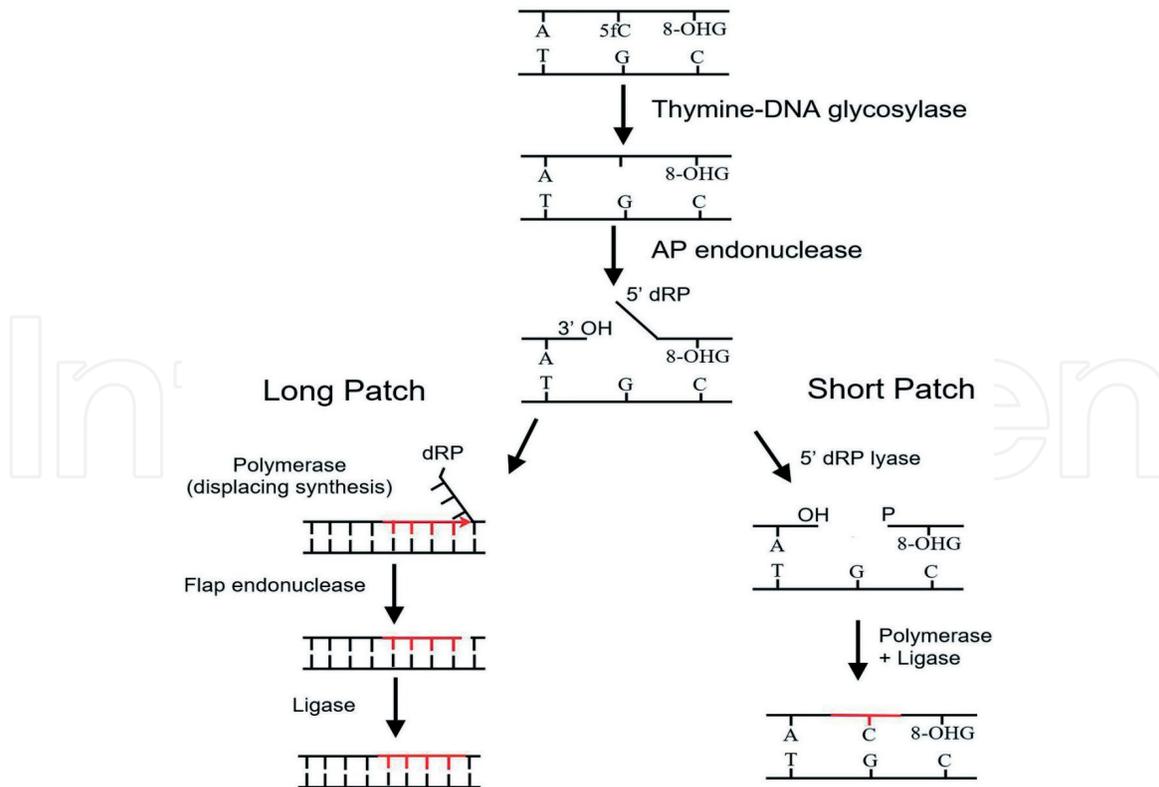


Figure 4.
An example of base excision repair of 5-formylcytosine (5fC) (adjacent to 8-OHG, an oxidized guanine) by short patch repair or long patch repair.

4. TET enzymes

As described by Jin et al. [27] and Melamed et al. [28], there are a number of TET enzymes, including at least two isoforms of TET1, one of TET2 and three isoforms of TET3. As reviewed [28], the full-length canonical TET1 isoform appears virtually restricted to early embryos, embryonic stem cells and PGCs. The dominant TET1 isoform in most somatic tissues, at least in the mouse, arises from alternative promoter usage which gives rise to a short transcript and a truncated protein designated TET1s. The isoforms of TET3 are the full length form TET3FL, a short form splice variant TET3s, and a form that occurs in oocytes and neurons designated TET3o. TET3o is created by alternative promoter use and contains an additional first N-terminal exon coding for 11 amino acids. TET3o only occurs in oocytes and neurons and was not expressed in embryonic stem cells or in any other cell type or adult mouse tissue tested [27]. Whereas TET1 expression can barely be detected in oocytes and zygotes, and TET2 is only moderately expressed, the TET3 variant TET3o shows extremely high levels of expression in oocytes and zygotes, but is nearly absent at the 2-cell stage [29].

The TET enzymes generally do not specifically bind to 5-methylcytosine except under particular conditions, such as the two conditions described below, in “Targeting TET1 to 5-methylcytosine” and in “TET in learning and memory.” Without targeting, TET1 predominantly binds to high CG promoters and CpG islands (CGIs) genome-wide by its CXXC domain that can recognize un-methylated CGIs [30]. TET2 does not have an affinity for 5-methylcytosine in DNA [31]. The CXXC domain of the full-length TET3, which is the predominant form expressed in neurons, binds most strongly to CpGs modified by 5-carboxycytosine (5caC) (Figure 3), although it does also bind to un-methylated CpGs [28].

4.1 Targeting TET to 5-methylcytosine

One mode of recruitment of a TET enzyme to 5-methylcytosine in DNA, in order to initiate demethylation, was investigated by Zhou et al. [32]. In this mode, recruitment was found to depend on ROS treatment of cells. This finding is significant because appropriate levels of ROS are known to be needed in both embryogenesis [6, 7] and in learning and memory [8, 9]. ROS cause oxidative damages to DNA, but these damages are not random. Because electron “hole” pausing at the sites of the lowest ionization potential increases the probability of stable adduct formation, DNA oxidation tends to be sequence dependent [19]. As reviewed by Ming et al. [19], cytosine methylation increases the reactivity of guanine bases in 5mCpG dinucleotides toward electrophiles and oxidants. This is likely due to the transmission of an electronic effect from the 5mC to its partner guanine through hydrogen bonding within the 5mC:G base pair. Ming et al. [19] experimentally showed that oxidation of guanines was enhanced within endogenously methylated 5mCpG dinucleotides.

There are many types of oxidative DNA damage, but the most common endogenous oxidative damage in DNA is 8-OHdG [33]. The molecular structure of 8-OHdG is shown as part of **Figure 5**. In **Figure 5**, the structure labeled in red as “8-OHdG” is a guanine with the oxidative damage, an added OH group at the 8 position of the pentane (5-sided) ring, shown in red. 8-OHdG can be experimentally increased in cells by treatment with Hoechst dye followed by micro-irradiation with 405 nm light [34]. The irradiation can be performed along a narrow line.

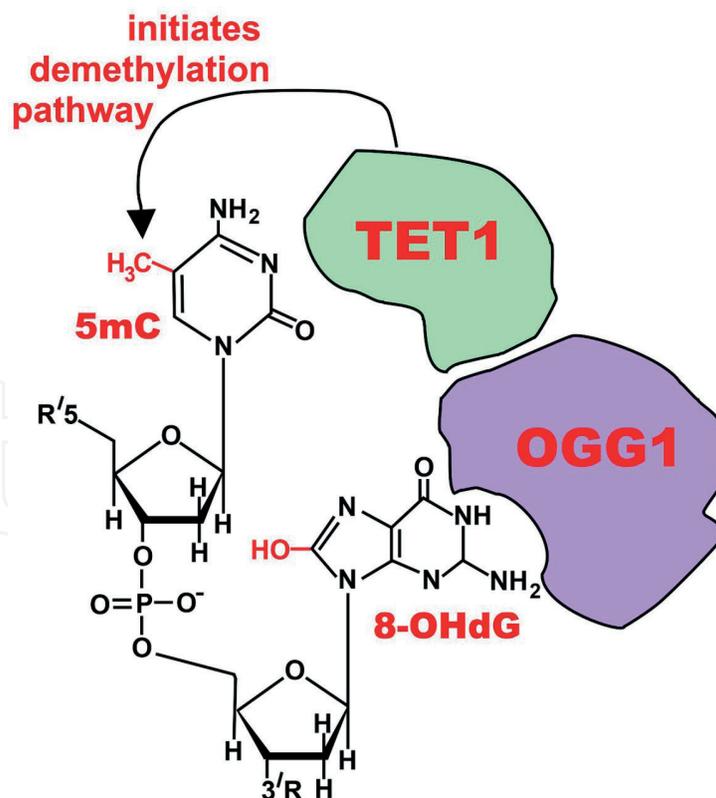


Figure 5.

Initiation of DNA demethylation at a CpG site. In adult somatic cells DNA methylation typically occurs in the context of CpG dinucleotides (CpG sites), forming 5-methylcytosine-pG, or 5mCpG. Reactive oxygen species (ROS) may attack guanine at the dinucleotide site, forming 8-hydroxy-2'-deoxyguanosine (8-OHdG), and resulting in a 5mCp-8-OHdG dinucleotide site. The base excision repair enzyme OGG1 targets 8-OHdG and binds to the lesion without immediate excision. OGG1, present at a 5mCp-8-OHdG site recruits TET1 and TET1 oxidizes the 5mC adjacent to the 8-OHdG. This initiates demethylation of 5mC [37].

Within 6 s of the irradiation with 405 nm light, there is half-maximum recruitment of OGG1 to the irradiated line. OGG1 (8-oxoguanine DNA glycosylase) is an enzyme that removes the oxidative damage 8-OHdG from DNA [35]. Removal of 8-OHdG, during base excision repair, occurs with a half-life of 11 min [36]. Thus, OGG1 protein rapidly complexes with 8-OHdG (6 s) but the OGG1-8-OHdG complex has a relatively long half-life (11 min).

H₂O₂ is a reactive oxygen species. Zhou et al. [32] treated cells in culture with 500 μM H₂O₂ for 6 h and this caused a more than 3-fold increase in 8-OHdG. The cells treated with H₂O₂ also became substantially demethylated, with methylation reduced to less than 1/4th the original methylation level. They then used cells in which OGG1 was inhibited, either by applying siRNA or by using OGG1 mutant knockout cells. In cells with inhibited or absent OGG1, treatment with H₂O₂ did not cause demethylation. These first experiments indicate that OGG1 has a role in H₂O₂-induced demethylation.

Zhou et al. [32] examined the interaction between OGG1 and the TET enzymes that are involved in demethylation [23]. OGG1 did not interact with TET2 or TET3. However, OGG1 interacted with TET1. They found that the two proteins co-immunoprecipitated, and this co-immunoprecipitation did not depend on interactions with DNA or with 8-OHdG. Thus, OGG1 can attract or “recruit” TET1. They then used a double-stranded oligonucleotide containing 8-OHdG in solution in a pull-down assay using streptavidin beads. They found that OGG1 added to the assay could be pulled down by oligonucleotides containing 8-OHdG. TET1 could not be pulled down by oligonucleotides containing 8-OHdG, but TET1 could be pulled down if in the presence of OGG1. Their results imply that OGG1 attaches to 8-OHdG and then recruits TET1 to 8-OHdG lesions. They indicated that this could allow TET1 to initiate DNA demethylation of methylated CpGs after 8-OHdG lesions are formed (**Figure 5**). As shown in this figure, TET1 first interacts with OGG1 and then is close enough to the methyl group CH₃ (shown in red) on the 5 position of the cytosine, to initiate the oxidation of the methyl group. This mechanism is notable for likely using two co-opted elements of DNA base excision repair (BER). First, OGG1 is an initiating enzyme in BER of 8-OHdG, but acts here to recruit TET1. Second, once the intermediate products of demethylation are formed by TET1, such as 5fC or 5caC as shown in **Figure 3**, then thymine DNA glycosylase (TDG) can initiate BER as shown in **Figure 4**, and complete the demethylation of 5mC to C.

OGG1 knockout mice seem to undergo a fairly normal embryogenesis, and the young new mice appear to be mostly normal [38], though they have a deficit in learning and memory as shown by a passive avoidance test [39] and a deficiency in immune responses (reviewed in [40]). TET1 knockout mice are also viable and fertile, with no discernible morphological or growth abnormality. However, TET1 knockout mice have an impairment in spatial learning and short-term memory [41] as well as deficiencies in fear memory extinction and spacial memory extinction [42]. On the other hand, over-expression of TET1 impairs hippocampus-dependent long-term associative memory [43]. A TET3 homozygous mutation, unlike a TET1 knockout, leads to neonatal lethality [44]. Thus TET3 is essential in embryogenesis. As pointed out above, TET3 (but not TET1 and TET2) is highly expressed in oocytes and zygotes (also shown in [45]).

5. Demethylation in neurogenesis

Neurogenesis in mouse takes place starting about day 10.5 after fertilization of the egg. Early in neurogenesis, some embryonic stem cells (ESCs) begin

differentiating into neural stem cells (NSCs) and neural progenitor cells (NPCs) [46]. At this point, 8% of CpGs unmethylated in ESCs become largely methylated in NPCs, whereas approximately 2% of CpGs methylated in ESCs become unmethylated [46]. These data suggest that 5mC undergoes significant dynamic changes during ESC differentiation into NSCs. As shown by Pilz et al. [47], NPCs generate neurons throughout life in the dentate gyrus of the hippocampus of mice. Zhang et al. [41] examined adult NPCs purified from wild type and TET1 knockout mice. They found that 478 genes showed elevated promoter methylation levels in TET1-null NPCs compared to the wild-type control, while only 32 genes had lower methylation. Thus, TET1 appears to function in demethylation during neurogenesis in the adult brain.

6. Demethylation in learning and memory

Learning and memory have levels of permanence, differing from other mental processes such as thought, language, and consciousness, which are temporary in nature. Learning and memory can be either slowly accumulated (multiplication tables) or rapidly (touching a hot stove), but once attained, can be recalled into conscious use for a long time. As pointed out by Alberini [48], humans can generally recall a painful fact or trauma in detail for a lifetime. Similarly, humans remember a very happy day for a long time afterwards. At least two early proposals were presented, indicating, on theoretical grounds, that the methylation and demethylation of DNA in neurons is the physical basis of memories. In 1969 Griffith and Mahler [49] published an article that made a number of salient points. They noted that, at least in man, memories may survive for periods of almost the entire lifetime. Further, DNA is the one molecule which, apart from possible minor effects due to genetic damage and repair, is surely present in neurons for the whole of the lifetime of the organism. This led them to the suggestion that the physical basis of memory could lie in the enzymatic modification of the DNA of nerve cells. They further indicated that a plausible suggestion would be that the modification consists of methylation (or demethylation) of DNA.

In 1999 Holliday [50] noted that long-term human memory can be retained for many decades. The exceptional stability required suggests that essential memory components may be based on chemical changes. He proposed that the enzymatic modification of cytosine in DNA to 5-methylcytosine may provide this necessary stability. The general model proposed is that specific sites in the DNA of neurons required for memory can exist in alternative methylated or non-methylated states. The initial signal, which is to be memorized, switches the DNA from a modified to an unmodified state, or vice versa. It should be noted that the presence or absence of DNA methylation at a particular sequence of DNA can be thought of as a 0, 1 binary code. Thus, 10 such sites have 2^{10} (1024) epigenotypes and potential phenotypes, and 30 such sites could have up to 2^{30} , or 1.07×10^9 epigenotypes. Clearly, such a set of control mechanisms has enormous potential for neuronal specificity.

One form of long-term memory, associative learning, is contextual fear conditioning [51]. As an example of contextual fear conditioning, a rodent is placed in a novel environment (a new context) and is then subjected to an electric shock (e.g. a footshock). The rodent then experiences robust fear learning, shown by a strong fear response, when the rodent is placed in that context again. Contextual fear conditioning occurs very rapidly (it can occur with a single event) and it has a lasting effect [51]. Kim and Jung [51] reviewed the evidence that the hippocampus region of the brain is where contextual fear memories are first stored, and that this storage is transient and does not remain in the hippocampus (**Figure 6**). (Note that

while this diagram shows a single hippocampus in a human brain, humans have two hippocampi, one in each hemisphere of the brain.) They point out, in rats, that contextual fear conditioning is abolished when the hippocampus is subjected to hippocampectomy just 1 day after conditioning. However, the rats retain a considerable amount of contextual fear when a long delay of 28 days is imposed between the time of conditioning and the time of hippocampectomy. Using localized lidocaine injections to impede brain functions, Frankland et al. [53] showed that much of the long term storage of contextual fear conditioning memory appears to take place in the anterior cingulate cortex (**Figure 6**) (Note that there is a single anterior cingulate cortex of the human brain and it *resides in the medial wall of the two cerebral hemispheres*).

When methods to detect DNA methylation at specific locations on chromosomes became available, early experiments focused on particular genes known to be important for memory. One such gene is *PP2B* (*protein phosphatase 2B*), also known as *calcineurin* (*CaN*). This gene is of particular interest because it is the only Ca^{++} -activated protein phosphatase in the brain and a major regulator of key proteins essential for synaptic transmission and neuronal excitability [54]. Miller et al. [55] found that persistent, specific hypermethylation of the *CaN* gene in the anterior cingulate cortex was induced in rats by a single contextual fear conditioning event at a time when a long-term memory was formed. Demethylation at a specific locus also has been investigated. Brain-derived neurotrophic factor (BDNF) is known to be important in memory [56]. As reviewed by Lubin et al. [57], the *bdnf* gene consists of eight 5' exons each linked to individual promoter regions, and a 3' exon (IX). Lubin et al. [57] subjected rats to contextual fear conditioning. Their sequencing data confirmed active demethylation of *bdnf* exon IV after fear conditioning along with a strong increase in expression of exon IV in the hippocampus at 2 h after fear conditioning. As noted above [51], the hippocampus region of the brain is where contextual fear memories are first stored, but this storage is transient. In the experiments of Lubin et al. [57] the RNA expression of exon IV of the *bdnf* gene returned to baseline level by 24 h after the fear conditioning.

More recently, methods became available to identify differentially methylated genes in entire genomes. In 2016, Halder et al. [58] used mice subjected to contextual fear conditioning and evaluated whole neuron genomes for differentially methylated genes and for differentially expressed genes. In one part of their study they looked at

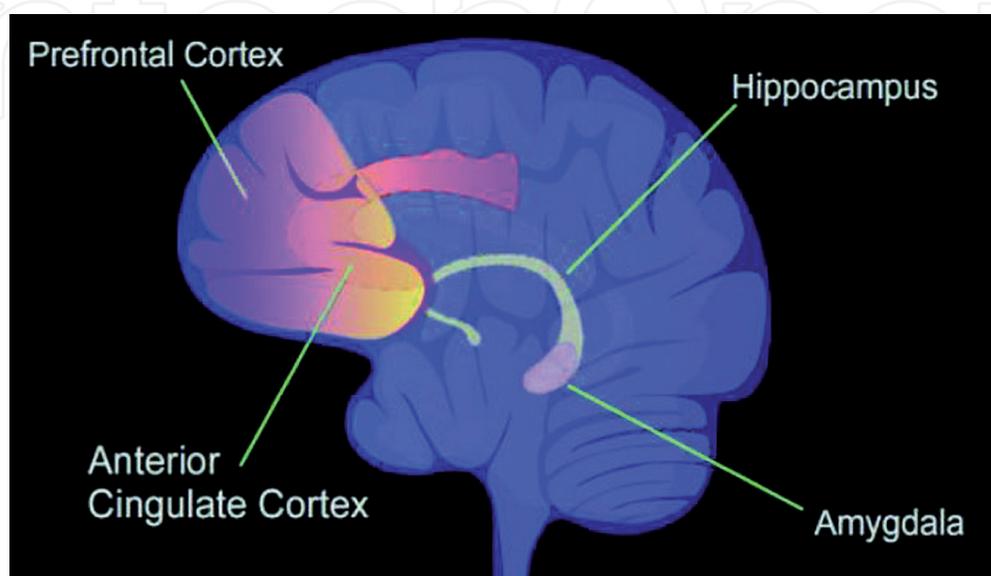


Figure 6.
Some regions of the brain involved in memory [52].

the hippocampal CA1 region, a region that is crucial for short-term memory formation during contextual fear conditioning. In the hippocampus 1 h after contextual fear conditioning, there were 675 demethylated genes and 613 hypermethylated genes. The consolidation of memory at 1 h after contextual fear conditioning was accompanied by the differential methylation of genes coding for ion channels, transcription factors, and constituents of the CREB and PKA signaling cascades, all of which have been shown to contribute to the early phases of learning and memory processes. These changes were transient in the hippocampal neurons, and almost none were present after 4 weeks. This also implies that the hypermethylated genes at 1 h then underwent active demethylation during the 4 weeks after contextual fear conditioning. Halder et al. [58], in addition, examined the anterior cingulate cortex, a brain region important for associative memory acquisition and maintenance of long-term memory. In the anterior cingulate cortex, at 1 h after contextual fear conditioning, there were 6250 differentially methylated genes, including 2423 demethylated genes. At 4 weeks after training 1223 differentially methylated genes persisted, including 118 demethylated genes. In addition, at 4 weeks after training they found 1700 differentially expressed genes in the anterior cingulate cortex. Their findings suggest that long-term memory (4 weeks) is associated with differential methylation of DNA and altered expression of more than a thousand genes in mouse neurons.

In 2017, Duke et al. [59], working with rats, studied neuron genomes in the hippocampus after contextual fear conditioning. At 24 h after contextual fear conditioning there were 2097 differentially methylated genes, with about 40% being demethylated. There were also 564 genes with upregulated expression and 1048 genes with downregulated expression. Hypermethylated regions overlapping differentially expressed genes were associated with decreased gene expression, consistent with the concept that cytosine methylation is often a mechanism for suppressing transcription. At 24 h after training, 9.2% of the genes in the rat genome of hippocampus neurons were differentially methylated. Gene Ontology term analysis was performed, and differentially expressed gene enrichment analysis revealed that many of the genes involved in synaptic functions were up-regulated 24 h after contextual fear conditioning in rats.

6.1 TET in learning and memory

In 2011, Guo et al. [26] were the first to show that TET1 is involved in neuronal activity-induced DNA demethylation and increased expression of memory-related genes in the mouse hippocampal dentate neurons. Demethylation of neuronal genes by TET1 appears to depend on TET1 being recruited to relevant genes. One mechanism of recruitment of TET appears to be by complexing with a specific “immediate early gene.” The immediate early genes (IEGs) are a class of genes that are rapidly and transiently activated by a variety of signaling cascades and phosphorylation events, usually in a protein synthesis-independent manner, in response to neuronal activation [60]. ERG1 (Krox-24, Zif268) is an IEG product and is a neuronal activity-induced transcription factor. ERG1 appears to play an important role in learning and memory [60]. ERG1 is required specifically for the consolidation of long-term memory (while the related transcription factor ERG3 is primarily essential for short-term memory). As reviewed by Sun et al. [61], the short form of TET1, TET1s, is present in the brain. Sun et al. [61] experimentally showed that EGR1 and TET1s form a complex, independently of attachment to DNA. ERG1 undergoes rapid induction and appears to attach to binding sites at many genes upon neuronal activation. When ERG1 binds to a site, it is able to recruit a TET1s enzyme to that site. This allows TET1s to cause demethylation of a gene downstream of the binding site of EGR1, with upregulation of that gene’s expression.

TET1 knockout mice [62] and *ERG1* knockout mice [63] are viable. Both have some developmental deficiencies [62, 63], and *TET1* knockouts [41, 43] and *ERG1* knockouts [64] each have some learning and memory deficiencies. Sun et al. [61] examined where differentially methylated regions occurred in the two types of knockout mice. Compared to wild-type mice, 322 and 2373 differentially methylated regions were identified in the brain frontal cortices (**Figure 6**) of *EGR1* knockout and *TET1* knockout mice respectively. There were 184 of these differentially methylated regions overlapping in the two types of knockout mice. This indicated that while *ERG1* can bring *TET1* to a DNA site to promote demethylation, *TET1* is also brought too many other sites as well, presumably by other factors.

7. Conclusions

In evolutionary biology, the term exaptation refers to an evolutionary shift in the function of a trait over the course of natural selection [65]. For instance, a trait may evolve initially because it serves a particular function, but during the course of further evolution it may come to serve another function or an additional function. Such shifts in function are thought to be common in evolutionary history. As one example, bird feathers likely evolved initially for temperature regulation, and were later adapted for flight [65].

The idea that the function of a trait may shift during evolution was for many decades referred to as “preadaptation”. However, this term suggests teleology in biology in conflict with natural selection and thus the term “preadaptation” has been replaced in the literature by “exaptation.” This concept has recently been applied to the cognitive neurosciences [66]. It was proposed that substantial changes in function such as development of contemporary complex cognition including grammatical language, reading, writing and calculation abilities have occurred without evident changes in brain morphology over the past 150,000 years.

The evolutionary emergence of embryonic development also appears to have depended on an early exaptation. Enzymatic pathways that repair damage to the DNA genome likely existed very early in the history of life [67]. Processes that repair DNA, such as base excision repair, can also facilitate epigenetic modifications, particularly demethylation reactions, that alter gene expression and hence the function of cell lineages. Such epigenetic modifications play a central role in embryonic development including neurogenesis. Epigenetic alterations such as 5-methylcytosine are structurally similar to unwanted damages that are the primary target of DNA repair processes. Thus acquiring the new function of recognizing epigenetically methylated bases may have been enabled by this similarity. However, in the case of epigenetic demethylations, the effect of removing methyl groups and restoring the genome is to allow expression of genes that had been previously epigenetically silenced by methylation. Methylation and demethylation are reciprocal processes that appear to act coordinately to direct gene expression during embryonic development. DNA methylation reactions often cause silencing of gene expression, while demethylation reactions can reverse this process to allow expression. These mechanisms for controlling gene expression and the consequent facilitation of cell differentiation leading to embryonic development may have emerged in evolution as early as the origin of multicellular organisms more than 1 billion years ago [68].

Just as the evolutionary shift in the function of DNA repair appears to be central to the emergence of embryonic development and neurogenesis, this derived capability likely also gave rise to memory and learning. The molecular processes of epigenetic methylation and demethylation that underlie embryonic development

also appear to underlie memory and learning. Thus the capacity for memory and learning may have evolved from a set of earlier epigenetic capabilities whose function was to promote embryonic reprogramming and neurogenesis.

In several neurodegenerative diseases epigenetic alterations appear to underlie characteristic features of the disease phenotype [69]. Proper functioning of the nervous system likely depends on DNA repair processes that not only restore DNA sequence information, but also facilitate normal gene expression by maintaining an appropriate set of epigenetic markers, particularly DNA methylation patterns. Understanding changes in DNA methylation patterns during early development and neurogenesis may contribute to the prevention or treatment of particular neurodegenerative diseases.

Parkinson disease patients treated with levodopa are subject to dyskinesia, a persistent behavioral sensitization that develops after levodopa exposure. Reorganization of DNA methylation patterns in the genome due to aberrant expression of DNA demethylation enzymes appears to have a pivotal role in the development of levodopa-induced dyskinesia [70]. Modification of DNA methylation is considered to be a promising novel therapeutic target for use in preventing or reversing dyskinetic behaviors [70]. Huntington's disease is a neurodegenerative disease that typically becomes apparent in midlife. This disease is associated with substantial changes in brain DNA methylation levels [71]. Aicardi-Goutieres syndrome (AGS) is a neurodegenerative condition characterized by early onset, often in infancy. Cells deficient in AGS proteins display a substantial 5–20% reduction in genomic methylation levels overall, and this reduction is distributed widely in the genome [72]. The fragile X syndrome is a prevalent form of mental retardation. This condition is caused by loss of expression of the *FMR1* gene, usually due to expansion of a CGG repeat sequence (>200 repeats) in the first exon of *FMR1*. This sequence expansion leads to abnormal methylation of the promoter region that then causes transcriptional silencing of the *FMR1* gene and an absence of the fragile X mental retardation protein [73]. Several studies have described methylation alterations in various regions of the brain in Alzheimer's disease, as reviewed by Yokoyama et al. [74]. The results of these studies, so far, appear to be somewhat contradictory and additional studies will be needed to provide clear conclusions. These various studies of DNA methylation alterations are still at an early stage, but nevertheless suggest that as our basic understanding of how epigenetic DNA methylation patterns influence neurodegenerative disease advances, this understanding will contribute to disease prevention and treatment.

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