

Focus: Molecular Memory

Mechanisms of epigenetic memory and addiction

Luis M Tuesta^{1,2,3} & Yi Zhang^{1,2,3,4,*}

Abstract

Epigenetic regulation of cellular identity and function is at least partly achieved through changes in covalent modifications on DNA and histones. Much progress has been made in recent years to understand how these covalent modifications affect cell identity and function. Despite the advances, whether and how epigenetic factors contribute to memory formation is still poorly understood. In this review, we discuss recent progress in elucidating epigenetic mechanisms of learning and memory, primarily at the DNA level, and look ahead to discuss their potential implications in reward memory and development of drug addiction.

Keywords addiction; DNA methylation; epigenetics; histone modifications; learning & memory

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See the Glossary for abbreviations used in this article.

Introduction

The term ‘epigenetics’ refers to heritable changes in phenotype or gene expression that cannot be directly attributed to changes in DNA sequence (Jaenisch & Bird, 2003). In eukaryotes, DNA and histones are assembled into nucleosomes, the basic unit of chromatin. Nucleosomes are composed of 147 DNA base pairs wrapped around an octamer of four core histone proteins: H2A, H2B, H3, and H4 (Kornberg & Lorch, 1999). Histones contain extensive post-translational modifications that regulate their various functions (Kouzarides, 2007). Similarly, DNA methylation at CpG dinucleotides can also modulate transcriptional outcome (Jaenisch & Bird, 2003). Some of these modifications are heritable and are believed to be responsible for a number of phenomena such as genomic imprinting, paramutation, Polycomb silencing, and position effect variegation (Bird, 1986; Campos & Reinberg, 2009; Fedorova & Zink, 2008; Martin & Zhang, 2007).

Epigenetic regulation has been mostly studied in the context of cellular differentiation and development, but accumulating evidence suggests that epigenetic mechanisms also play important roles

throughout the lifespan of postmitotic cells. As such, epigenetic changes may also regulate mechanisms underlying learning and memory, including brain reward and the development of drug addiction. In this review, we will cover the various types of epigenetic modifications on DNA and histones. We will also address the role of epigenetic modifications in postmitotic neurons, in the context of brain learning and memory. Finally, we will review recent progress in understanding the epigenetic regulation of drug addiction that sets the stage for future research in this burgeoning field.

DNA methylation

DNA methylation is the best-characterized form of epigenetic modification. It takes place at the 5' position of cytosine and usually occurs in the context of CpG dinucleotides. However, contiguous groups of CpG dinucleotides, called ‘CpG islands’, are generally unmethylated. Promoter methylation is generally associated with gene silencing either by preventing binding of transcription factors or by attracting methyl-CpG binding proteins that recruit co-repressors of transcription (Bird, 1986; Klose & Bird, 2006).

DNA methylation plays important roles in several physiological phenomena (Jaenisch & Bird, 2003). For example, genomic imprinting, an allele-specific expression phenomenon, is controlled by allele-specific DNA methylation. Additionally, X-chromosome inactivation, a mechanism used to equalize X-linked gene expression in males and females, uses DNA methylation to silence one of the two female X chromosomes. DNA methylation also plays a crucial role in other cellular processes such as cell differentiation and tissue-specific gene expression. CpG island methylation is commonly detected in tissue-specific and germline-specific genes, X-linked genes, and imprinted genes (Jaenisch & Bird, 2003).

DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which are classified into *de novo* and maintenance DNMTs based on their substrate preference (Goll & Bestor, 2005). *De novo* DNMT3A and DNMT3B prefer unmethylated DNA substrates, while DNMT1 prefers hemimethylated DNA substrates and is mainly responsible for copying the DNA methylation pattern during DNA replication (Hermann *et al*, 2004; Inano *et al*, 2000; Moore *et al*, 2013). Interestingly, DNMT3A is abundantly expressed in the postnatal brain (Feng *et al*, 2005), which suggests that it may also play a regulatory role in postmitotic neurons.

DNA methylation is relatively stable when compared to histone modifications, yet DNA demethylation has also been observed in

1 Howard Hughes Medical Institute, Boston, MA, USA

2 Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA, USA

3 Department of Genetics, Harvard Medical School, Boston, MA, USA

4 Harvard Stem Cell Institute, Harvard Medical School, Boston, MA, USA

*Corresponding author. Tel: +1 617 713 8666; Fax: +1 617 713 8665; E-mail: yzhang@genetics.med.harvard.edu

Glossary

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
BDNF	brain-derived neurotrophic factor
BER	base excision repair
DA	dopamine
DNMT	DNA methyltransferase
GABA	gamma-aminobutyric acid
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
HMT	histone methyltransferase
LTP	long-term potentiation
NAC	nucleus accumbens
PFC	prefrontal cortex
SNpc	substantia nigra pars compacta
TDG	thymine-DNA glycosylase
TET	ten-eleven translocation
VTA	ventral tegmental area

various biological contexts by active and passive means. Active DNA demethylation involves enzymatic activity that selectively restores an unmodified cytosine base, while passive demethylation generally involves dilution of 5-methylcytosine (5mC) through progressive cell division where DNA methylation maintenance machinery is either absent or compromised (Moore *et al.*, 2013; Ooi & Bestor, 2008). Until recently, a mechanism underlying active DNA demethylation has remained a topic of controversy.

It has now been shown that ten-eleven translocation (TET) family proteins can catalyze oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He *et al.*, 2011b; Ito *et al.*, 2010, 2011; Kriaucionis & Heintz, 2009; Tahiliani *et al.*, 2009). Thymine DNA glycosylase (TDG), an enzyme previously known for its role in DNA repair, has been shown to excise 5fC and 5caC, creating abasic sites which can be repaired by the base excision repair (BER) pathway to generate an unmodified cytosine residue, thereby completing the demethylation process (He *et al.*, 2011b; Shen *et al.*, 2013; Zhang *et al.*, 2012) (Fig 1). Interestingly, 5hmC, the major oxidation product of 5mC, positively correlates with transcription when it is located in the gene body, and is at its highest levels in neurons (Jin *et al.*, 2011; Khare *et al.*, 2012; Kriaucionis & Heintz, 2009; Szulwach *et al.*, 2011).

Given the abundance of 5hmC in neurons, the discovery of this new cytosine modification is especially exciting as it suggests that neural systems may be under active TET regulation, independent of cell division and maturation, opening the possibility for epigenetic regulation of adaptive processes such as learning and memory.

Histone modifications

In addition to DNA methylation, covalent modifications on histones also play an important role in regulating gene expression. Major histone modifications include acetylation, methylation, ubiquitination, and phosphorylation (Bannister & Kouzarides, 2011). These modifications can either activate or repress transcription, depending on the modification and the specific substrate residues (Margueron *et al.*, 2005; Martin & Zhang, 2005; Zhang, 2003), and can be

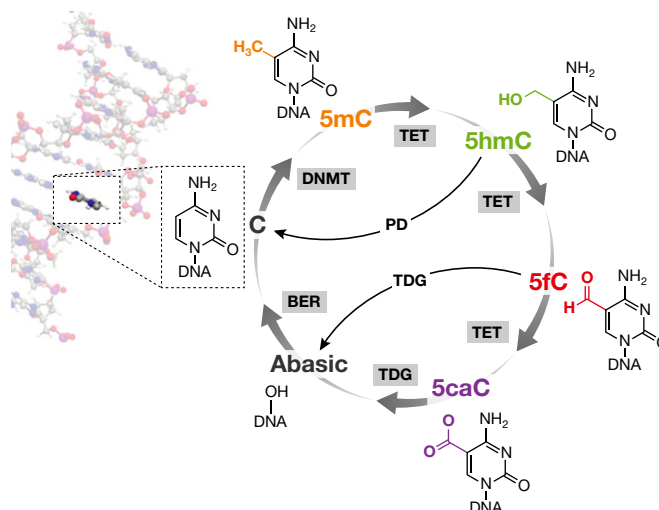


Figure 1. TET/TDG-mediated 5mC demethylation.

Unmodified cytosine residues (C), highlighted left, are methylated by DNMTs to produce 5mC. TET enzymes catalyze oxidation of 5mC to 5hmC (Ito *et al.*, 2010; Tahiliani *et al.*, 2009) and further to 5fC and 5caC (Ito *et al.*, 2011). In this pathway, 5mC demethylation can occur through passive dilution (PD) of 5hmC, or through active excision of TET oxidation products 5fC and 5caC to generate abasic sites via TDG that are then restored to unmodified C by BER (He *et al.*, 2011b; Shen *et al.*, 2013; Zhang *et al.*, 2012).

deposited or removed by a large family of histone-modifying proteins. While DNA methylation is faithfully inherited through semi-conservative replication (Bestor, 1992; Holliday & Pugh, 1975; Leonhardt *et al.*, 1992), the mechanisms by which epigenetic information is inherited through histone modifications still remain unresolved.

Histone acetylation, which occurs at certain lysine (K) residues of histones H3 and H4, is one common form of histone modification associated with transcriptional activation. Upon acetylation, chromatin is generally decondensed due to the neutralization of the positively charged K residues in histone tails. Acetylation and deacetylation of K residues are mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively (Borrelli *et al.*, 2008), yet the specificity of HDACs and HATs for specific K residues still remains poorly understood. Despite a general correlation between histone K acetylation and transcriptional activity, such general correlation seems to not hold true in brain at several promoters of genes involved in learning and memory, such as *Bdnf*, following chronic cocaine treatment, implying the involvement of alternate and complementary mechanisms of transcriptional regulation (Kumar *et al.*, 2005; Renthal *et al.*, 2009).

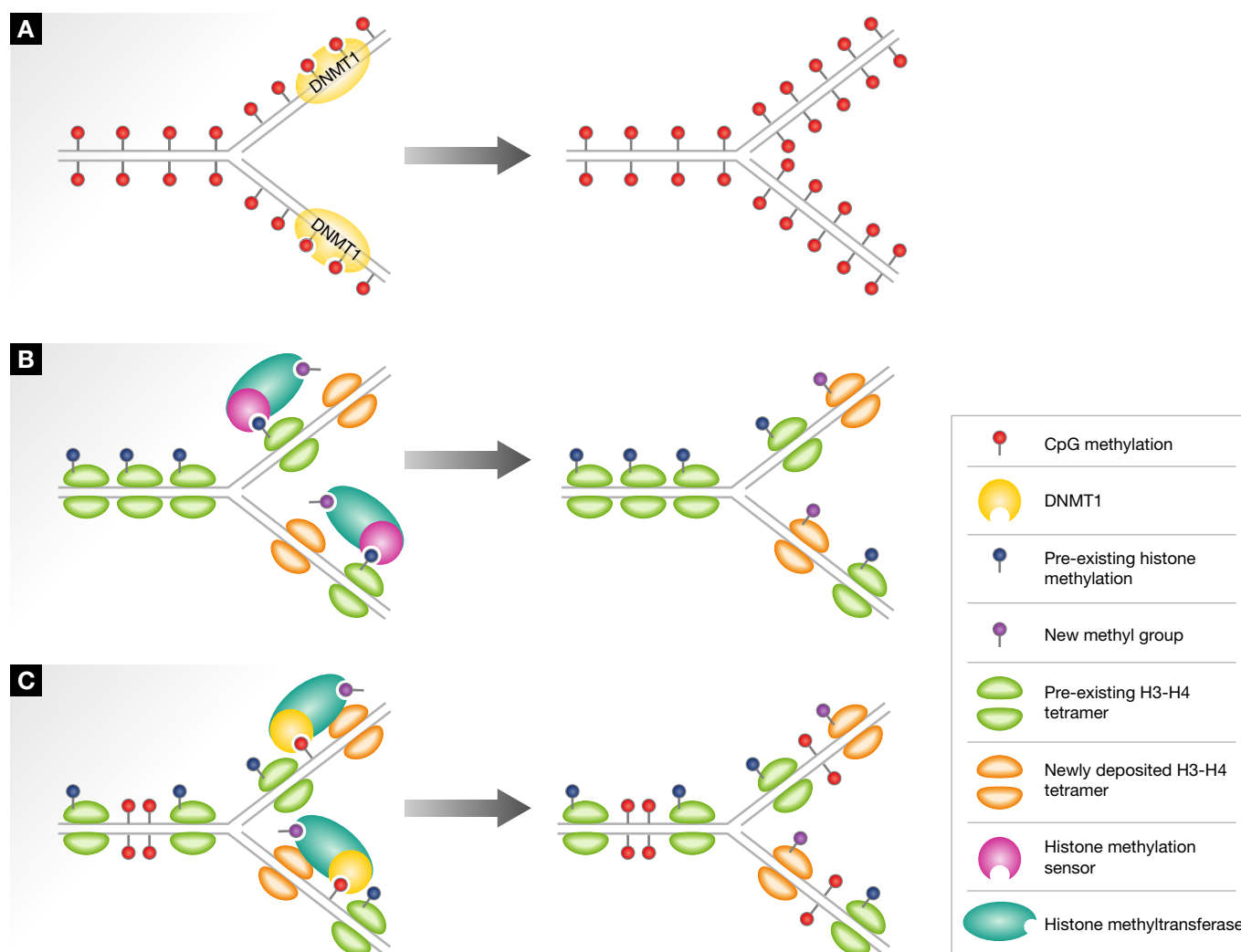
Histone methylation has been associated with both transcriptional activation and repression depending on the specific K residue and valence of methylation (Martin & Zhang, 2005). However, unlike acetylation, histone methyltransferases (HMTs) and histone demethylases (HDMs) have greater residue specificity, with distinct HMTs or HDMs acting on specific residues, determining valence of methylation states (mono-, di-, or trimethylation). Transcriptional silencing is mainly associated with H3K27me3 and H3K9me, whereas transcriptional activation is associated with H3K4me3 and H3K36me3 marks. Additionally, methylation marks can act as recruiters for other effector proteins that assist in perpetuating transcriptional states. Furthermore, some methyltransferases have

the ability to bind methylated DNA and certain transcriptional activators such as CREB-binding protein (CBP) (Volkel & Angrand, 2007).

As mentioned above, HDMs possess greater functional specificity than HDACs and they belong to the LSD1 and the JmjC family of proteins (Klose *et al*, 2006). Histone demethylation appears to occur in a gene-specific manner, in part by conjunction with nuclear factor complexes (Metzger *et al*, 2005; Tsukada *et al*, 2006; Yamane *et al*, 2006). Despite this, similar to histone acetylation, methylation states at certain gene promoters also fail to faithfully predict transcriptional behavior in brain following drug treatment (Renthal *et al*, 2009). This indicates that the transcriptional effects can be modulated by other factors and consequently one cannot predict transcriptional activity solely based on histone modifications.

Mechanisms of epigenetic inheritance

Classical 'epigenetic' modifications require a component of heritability. In the context of this definition, DNA CpG methylation has the best understood mechanism. As depicted in Fig 2A, CpG methylation is copied in a semi-conservative fashion where DNMT1 is recruited by UHRF1 and PCNA to the replication fork and reestablishes methylation marks on newly synthesized DNA strands (Zhu & Reinberg, 2011). In a similar fashion, chromatin modifiers have been reported to localize to replication forks (Esteve *et al*, 2006; Milutinovic *et al*, 2002), raising the possibility that inheritance of histone modifications is an opportunistic phenomenon that utilizes DNA replication as an avenue to rapidly pass on epigenetic information. While histone modifications are a major source of heritable epigenetic information, the exact modifications, degree of fidelity, and mechanisms of inheritance still remain a subject of investigation.



In the context of inheritance, K methylation has garnered special interest because of its relative stability (Huang *et al*, 2013). While histone lysine methylation marks possess a half-life ranging from hours to days (Zee *et al*, 2010), histone acetylation and phosphorylation marks are much more short-lived, with half-lives in the range of minutes (Chestier & Yaniv, 1979; Jackson *et al*, 1975). As such, histone K methylation has become a favored modification in the study of mechanisms of epigenetic inheritance. Based on the relationship to CpG methylation, several models for inheritance of histone modifications have been proposed (Xu *et al*, 2012; Zhu & Reinberg, 2011).

Conservative model of chromatin assembly As mentioned previously, CpG methylation occurs in a semi-conservative fashion where DNMT1 is recruited to the replication fork, methylating CpGs on nascent DNA strands. This mechanism prompted the proposal of a semi-conservative model of chromatin assembly (Weintraub *et al*, 1976). This model purported replication-dependent dissociation of H3-H4 tetramers into two half nucleosomes that would be deposited into each daughter strand. This was later proven incorrect by sedimentation studies using heavy isotope-labeled histones showing that nucleosomes do not divide, supporting conservative segregation of histone octamers during replication (Lefkay *et al*, 1977). However, speculation of a semi-conservative model was refuted when it was discovered that H3-H4 tetramers deposit onto chromatin, not as tetramers, but as dimers (Benson *et al*, 2006; English *et al*, 2005; Tagami *et al*, 2004), perhaps as a result of Asf1-mediated inhibition of H3-H4 tetramer formation (English *et al*, 2006). Regardless, more recent work suggests otherwise in that H3-H4 tetramers indeed do not divide and in fact, also suggests that inheritance of histone modifications occurs by copying modifications from neighboring pre-existing nucleosomes (Xu *et al*, 2010). This brings about an established conservative model of chromatin assembly in which H3-H4 tetramers are transferred intact to daughter strands and serve as methylation templates for *cis* nucleosomes (Martin & Zhang, 2007; Zhu & Reinberg, 2011) (Fig 2B). This raises the question of how modifications on 'old' histones are transferred to 'new' adjacent histones. The answer to this question remains under debate.

Templated modification One mechanism used to spread histone modifications involves the coupling of a chromatin-modifying enzyme to an effector protein that recognizes specific epigenetic marks, thereby allowing propagation of a modification state (Zhu & Reinberg, 2011). For example, recognition of H3K27me3 by Polycomb repressive complex 2 (PRC2) promotes propagation of this repressive signal onto neighboring histones through allosteric activation of its catalytic domain (Margueron *et al*, 2009) (Fig 2B). This suggests that histone modifications may be copied from 'template' histones that use their existing modifications as molecular flags to attract chromatin-modifying enzymes and propagate epigenetic states.

Modification reinforcement As mentioned earlier, chromatin-modifying enzymes, such as DNMT1, can be recruited to replication foci. This raises the possibility that replication-dependent DNA methylation can be coupled to histone modification. In fact, DNMT1 directly interacts with, and recruits G9a, an H3K9 methyltransferase,

to replication foci and regulates G9a-dependent H3K9 methylation (Esteve *et al*, 2006). The fact that knockdown of DNMT1 impairs H3K9 methylation suggests that inheritance of histone modifications may involve an opportunistic mechanism where factors such as G9a 'piggyback' on DNA replication factors to rapidly restore histone modifications on nascent chromatin (Fig 2C).

Histone maturation Some histone marks, such as H3K9 dimethylation reach similar levels in new histones as those in old histones shortly after S phase, suggesting a replication-dependent mechanism (Xu *et al*, 2012). However, other modifications such as H4K20, and H3K79 methylation, as well as H3K9 and H3K27 trimethylation, are gradually restored throughout the cell cycle, independent of replication (Pesavento *et al*, 2008; Sweet *et al*, 2010; Xu *et al*, 2012). This gradual 'maturation' of histone methylation suggests that epigenetic inheritance is not a rigid process, and its inexact fidelity provides a certain degree of epigenomic flexibility to adapt to changing conditions.

Brain memory and long-term potentiation

While the mechanisms outlined above largely rely on replication, it is unclear to what extent these mechanisms may play in postmitotic neurons, especially in the context of learning, memory, and addictive disorders.

A fundamental tenet of brain learning and memory postulates that neuronal activity has the ability to either strengthen or weaken connections at the synapse, the anatomical interface where direct communication between neurons occurs. This adaptability is referred to as synaptic plasticity, and the underlying foundation of activity-dependent synaptic plasticity is measured by long-term potentiation (LTP). Experience-dependent LTP involves structural changes such as dendritic spine remodeling and receptor redistribution, resulting in long-term increases in efficacy of synaptic transmission between neurons (Bliss & Collingridge, 1993; Luscher & Malenka, 2012; Toni *et al*, 1999). As such, LTP represents an integral component of learning and memory.

Fear conditioning is a widely used behavioral paradigm to measure associative learning in animals, where a neutral conditioned stimulus elicits a fear response (usually freezing behavior) following repeated pairing with a noxious, unconditioned stimulus such as a loud noise or a mild electrical foot shock (Kim & Jung, 2006). Indeed, fear conditioning via electrical foot shock has been shown to induce LTP in the amygdala of rats (Rogan *et al*, 1997). In addition to fear conditioning, reward learning in the form of active cocaine self-administration has also been shown to elicit LTP in brain regions implicated in drug reward (Chen *et al*, 2008).

It is interesting to note that a memory can be perpetuated throughout the lifetime of an individual, yet conventional molecular players in LTP formation such as CaMKII do not appear to be involved in lifelong maintenance of a memory (Day & Sweatt, 2011), raising the possibility for alternative mechanisms such as epigenetic modifications in mediating memory maintenance.

Epigenetic mechanisms underlying learning and memory

Cellular differentiation and phenotypic manifestation is heavily influenced by epigenetic mechanisms. However, as discussed in the

previous section, classical 'epigenetic' mechanisms involve some aspect of cell division. Stepping outside the realm of epigenetic inheritance in mitotic cells, the adult brain consists primarily of glia and postmitotic neurons, with very limited potential for proliferation (Emsley *et al*, 2005). If the cellular epigenetic toolbox is not utilized for the purpose of cell division or fate determination, another function must justify active regulation seen in these cells. Accumulating evidence suggests that epigenetic mechanisms play an important role in the formation and maintenance of memory in the brain.

Histone acetylation in learning and memory

Initial studies into the role of histone acetylation in learning and memory showed that MAP kinase (MAPK) activity regulates the formation of taste aversion in mice and that this in turn regulates histone acetylation in the insular cortex, a brain region associated with emotional processing and aversion memory (Swank & Sweatt, 2001). Further studies confirmed a role of histone acetylation in learning and memory. For instance, pharmacological inhibition of HDACs has proven to restore deficits in neuronal plasticity and fear memory in animals lacking the histone acetyltransferase CBP (Alarcon *et al*, 2004). Indeed, deficits in CBP expression are associated with impaired long-term fear memory and object recognition (Wood *et al*, 2006a,b). Furthermore, the HDAC inhibitor sodium butyrate (NaBut) significantly reduces the amount of training required for an animal to remember a novel object and prolongs the time the animal remembers the object following training (Stefanko *et al*, 2009). Another recent study also suggests that NaBut administration can improve retrieval of long-term inaccessible fear memories (Fischer *et al*, 2007). NaBut inhibits HDAC-mediated histone deacetylation, likely making DNA more accessible to transcriptional control, implying that histone acetylation plays a regulatory role in memory formation. In fact, aspects of memory formation and storage have shown to be mediated by several HDAC subtypes (Bahari-Javan *et al*, 2012; Guan *et al*, 2009; Kim *et al*, 2012; Sando *et al*, 2012). However, due to space limit, in the context of learning and memory, we focus our discussion on DNA methylation.

DNA methylation in learning and memory

DNA methylation has long been considered a relatively stable epigenetic mark. It is thus not surprising that efforts have been undertaken to explore the link between DNA methylation and learning and memory. Initial studies into the basic role of DNMTs in the brain suggest a role in DNA mismatch repair (Brooks *et al*, 1996), neuronal survival (Fan *et al*, 2001), and secondary neurodegeneration following ischemic insult (Endres *et al*, 2001, 2000). DNMT dysfunction has also been linked to cognitive and behavioral disorders such as schizophrenia (Veldic *et al*, 2004), fragile X syndrome (Sutcliffe *et al*, 1992), Rett syndrome (Amir *et al*, 1999), and aging-related cognitive decline (Oliveira *et al*, 2012).

The question is whether DNA methylation has a role in the induction of synaptic plasticity. As discussed earlier, learning and memory depends heavily on plastic adaptations between neuronal connections, where LTP enhances synaptic transmission efficacy, thereby facilitating development of memory formation. Indeed, inhibition of DNMT activity disrupts LTP in adult hippocampal slices, and inhibition of DNMT alters DNA methylation within the promoter regions of *reelin* and *BDNF*, two genes previously implicated in synaptic plasticity within the adult hippocampus (Levenson

et al, 2006). These experiments suggest a role for DNA methylation in memory formation by regulating LTP.

In vivo studies using a fear-conditioning model have shown that inhibition of DNMT enzymes in the hippocampus disrupts conditioned shock-fear memory formation and does not affect maintenance of the fear memory trace (Miller & Sweatt, 2007). This suggests that while the hippocampus is a key mediator of memory formation, there are alternative brain structures that can maintain a long-term memory trace long after cessation of the initial stimulus (Miller *et al*, 2010). Furthermore, when DNA methylation is disrupted in the dorsomedial prefrontal cortex (dmPFC) in mice, severe deficits in long-term fear memory consolidation are observed although short-term fear memory remains unaffected (Miller *et al*, 2010). These studies further suggest that while memory formation depends on hippocampal activity, consolidation and long-term maintenance of the memory trace occurs in cortical regions and that these mechanisms rely heavily on temporally discrete DNA methylation patterns.

It must be noted, however, that the studies outlined above (Miller *et al*, 2010; Miller & Sweatt, 2007) used 5-azacytidine (5-aza) and zebularine as pharmacological agents to inhibit DNA methylation in the brain and measure their effect on memory formation. Since 5-aza- and zebularine-mediated inhibition of DNA methylation requires their incorporation into DNA during replication (Szyf, 2009), it is mechanistically not clear how they could be incorporated into the postmitotic neurons to exert their effect on DNA methylation. Hypothetically, 5-aza may compete with cytosine for incorporation into neuronal DNA through a base excision repair (BER) mechanism if fear conditioning introduces DNA damage, but it is yet unclear how such a mechanism would contribute to the regulation of memory formation or whether the observed effects are due to a secondary effect of the small molecule. Regardless of how 5-aza and zebularine mediate the effects, the authors were able to confirm that DNMT inhibition indeed does disrupt long-term memory (Miller *et al*, 2010) using a non-nucleoside DNMT inhibitor, RG108, whose function in inhibiting DNA methylation does not require DNA replication (Brueckner *et al*, 2005).

The majority of studies into the role of DNA methylation on cognition using *in vivo* approaches mainly employ pharmacological techniques (intracranial infusions) to inhibit DNMT enzymes in animals. Consequently, these studies cannot definitively link the observed effect to a specific DNMT isoform. Therefore, a major challenge in the field involves dissecting the functions of individual epigenetic modifying enzymes and how they contribute to learning and memory process. To begin to address this issue, studies using mice lacking *Dnmt1*, *Dnmt3a*, or both showed that learning deficits are only present in animals lacking both isoforms, but not in single KO animals (Feng *et al*, 2010), suggesting some level of functional redundancy between these two DNMT enzymes. Although we still do not know exactly how DNA methylation functions to promote and maintain memory, characterization of DNA methylation patterns in the brain following stimulation (Guo *et al*, 2011a; Ma *et al*, 2009) may shed light on this question.

DNA demethylation in learning and memory

Given that the DNA methylation level is controlled by the concerted action of DNMTs and the demethylation machineries, it

is not surprising that learning and memory is also linked to loss of DNA methylation at certain genes. A recent study has shown that the offspring of mice conditioned to fear the odor of acetophenone (followed by electric foot shock) display greater behavioral sensitivity to the odorant, but not other odors (Dias & Ressler, 2014). Interestingly, fear conditioning results in hypomethylation of the *Olfir151*, a gene specific for the acetophenone odorant receptor, in fear-conditioned males as well as in their naïve progeny (Dias & Ressler, 2014). These results suggest that loss of DNA methylation caused by a traumatic experience can be inherited to subsequent generations. Although most of the studies on the role of DNA methylation in learning and memory have been focused on DNMTs, the recent identification of the DNA demethylation pathway has provided a new angle by which to study epigenetic changes involved in learning and memory (Kohli & Zhang, 2013; Wu & Zhang, 2011).

As discussed earlier, DNA demethylation can be achieved through TET-mediated oxidation followed by TDG-mediated cleavage and BER (Fig 1). Interestingly, TET oxidation product 5hmC accumulates at the highest level in the mammalian brain when compared to other tissues (Kriaucionis & Heintz, 2009; Szulwach *et al*, 2011) and has been proposed to act as a mediator of passive demethylation by interfering with DNMT1 (Smith & Meissner, 2013) as well as to function as a key intermediate of active demethylation (Fig 1). Considering that all the three TET proteins (TET1-3) are abundantly expressed in brain, they are believed to have important functions in postmitotic neurons. In fact, oxidation of 5mC to 5hmC by TET1 has been shown to promote DNA demethylation in the adult brain (Guo *et al*, 2011b). This study suggests the involvement of the AID/APOBEC family of cytidine deaminases in 5hmC demethylation, but more recent studies suggest that these deaminases favor unmodified cytosine (Nabel *et al*, 2012). Nevertheless, knockdown of endogenous TET1 in the dentate gyrus of the hippocampus can reverse the demethylation observed in the promoter region of *Bdnf* and *Fgf1* (Guo *et al*, 2011a) in animals that receive synchronous electroconvulsive therapy (Ma *et al*, 2009). In addition, overexpression of TET3 has been shown to disrupt anatomical targeting of neurons to the olfactory bulb (Colquitt *et al*, 2013), suggesting that TET proteins play a direct role in activity-dependent DNA demethylation and in the development of neuronal networks.

In addition to participating in neuronal DNA demethylation, recent *in vivo* studies utilizing TET1 KO mice have shown that global deletion of the protein impairs spatial learning and short-term memory in a Morris water maze (Zhang *et al*, 2013). However, another study has shown that deletion of TET1 affects long-term depression in the hippocampus, and while spatial memory and fear memory acquisition are unaffected, extinction of these is severely compromised (Rudenko *et al*, 2013). Despite conflicting reports of impaired spatial memory acquisition, both studies point to the fact that TET1 is a key regulator of memory extinction. A third report corroborates these findings, where fear conditioning, neuronal activation *ex vivo*, and seizure induction *in vivo* all reduce TET1 levels in the hippocampus, suggesting that TET1 is actively regulated by neuronal activity (Kaas *et al*, 2013). Interestingly, animals overexpressing TET1 in the dorsal hippocampus display impaired long-term fear memory formation, while short-term memory remains unaffected (Kaas *et al*, 2013),

indicating that homeostatic levels of TET1 are crucial for maintaining proper memory acquisition and consolidation.

TET enzymes catalyze DNA demethylation in brain and thereby regulate aspects of learning and memory. However, the underlying mechanism remains elusive unless we can identify specific genes involved in learning and memory that are susceptible to TET-mediated transcriptional regulation. In this regard, TET1 KO mice exhibit increased *Npas4* promoter methylation (Rudenko *et al*, 2013). *Npas4* is an activity-dependent transcription factor (Lin *et al*, 2008) that recruits RNA polymerase II to promoters of its target memory-associated genes, thereby allowing contextual memory formation. Importantly, *Npas4* deficiency has also been implicated in social cognitive regulation (Coutellier *et al*, 2012). Absence of TET1 decreases expression of *Npas4* and its target genes. Indeed, selective deletion of *Npas4* in the CA3 region of the hippocampus is sufficient to disrupt memory formation (Ramamoorthi *et al*, 2011), suggesting that, mechanistically, TET1 functions upstream of *Npas4* in determining its ability to regulate genes necessary for contextual memory formation. Looking forward, it would be interesting to test the effect of a CA3-specific TET1 knockdown on *Npas4* expression and contextual memory formation. This would circumvent confounds arising from any developmental compensation in a KO mouse and provide greater anatomical specificity for this type of regulation.

Epigenetic mechanisms underlying brain reward and drug addiction

Reward processing is an integral neural event that ensures survival in an organism, as it reinforces positive behaviors and experiences. Perception of a reward is mediated by the brain reward system and uses dopamine as its principal neurotransmitter. The ventral tegmental area (VTA) is a midbrain region crucial to reward processing and represents a major dopaminergic output in the brain. Dopaminergic output from the VTA primarily projects to the nucleus accumbens (NAc), a principal center for reward processing, while the dorsal striatum (DST), another region heavily implicated in addictive disorders, receives most of its dopaminergic input from midbrain neurons in the substantia nigra pars compacta (SNpc) (Fig 3) (Hyman *et al*, 2006; Russo & Nestler, 2013). Food reward has been shown to activate dopaminergic neurons in the VTA. Indeed, feeding behavior is heavily influenced by the expectation of pleasure and reward, and this proves to be a very powerful motivator of consumption (Saper *et al*, 2002; Zheng & Berthoud, 2007). A recent report suggests a role for DNA methylation in food reward. The study shows that associative learning for sucrose rewards increases methylation of learning-associated genes within dopaminergic neurons in the VTA and that inhibition of DNA methylation in this brain region, but not the NAc, prevents acquisition of the behavior (Day *et al*, 2013). This is the first report to link activity-dependent DNA methylation and demethylation to appetitive behavior such as volitional food reward consumption. Interestingly, it has also been reported that chronic overeating of a highly palatable diet leads to obesity that results in brain reward deficits as well as reduced D2R availability (Johnson & Kenny, 2010)—a phenomenon also seen in individuals with obesity (Volkow *et al*, 2008) and individuals with a history of cocaine abuse (Volkow *et al*, 1993). Just as

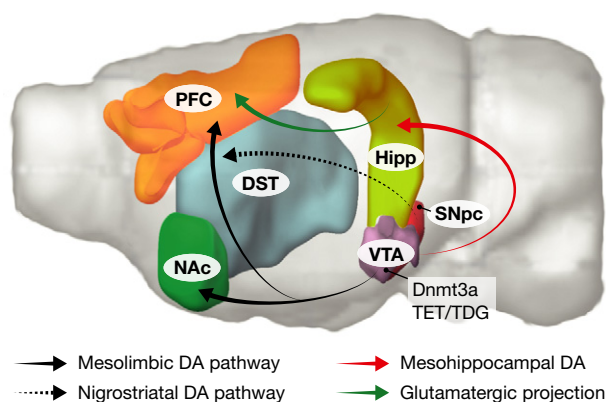


Figure 3. The dopaminergic reward pathway and epigenetic reward memory.

Dopamine (DA) output from the VTA primarily projects to the NAc and PFC (solid black), and nigral DA output primarily projects to the DST (dashed black) (Hyman *et al*, 2006; Russo & Nestler, 2013). However, the VTA also projects DAergic efferents (red) to the hippocampus (Hipp) (Scatton *et al*, 1980), composing the upper arm of the hippocampal-VTA loop (Lisman & Grace, 2005). Given the role of the hippocampus and PFC in memory processing, it is possible that DNMT/TET-driven epigenetic modifications in VTA DA neurons can regulate synaptic LTP within the hippocampus, thereby promoting long-term reward memory formation in the PFC. Image background of brain and corresponding structures are adapted from Brain Explorer 2 software, Allen Mouse Brain Atlas (Lein *et al*, 2007).

the midbrain dopamine system plays a major role in regulating food reward, drugs of abuse such as cocaine and nicotine have a very powerful effect on dopaminergic output (Kalivas *et al*, 1988; Nisell *et al*, 1994; Picciotto *et al*, 1998).

While natural rewards reinforce behaviors that help ensure survival in an organism, drugs of abuse do not. As opposed to food, drugs of abuse act directly on dopaminergic neurons, resulting in overactivation of brain reward circuitry. Indeed, development of addiction can be characterized by increased consumption, followed by a persistent desire for, and to acquire, the drug. As consumption further increases and becomes habitual, tolerance may develop, and a negative affective state akin to withdrawal will be established when the drug is unavailable. Finally, this cycle of behavior may not only affect physiological reward processing, but also severely compromise social and occupational activities of the individual (Koob & Le Moal, 2001). It should be noted, however, that casual drug use does not always result in transition to compulsive drug use and addiction. In fact, only about 17% of cocaine users eventually become addicted (Anthony *et al*, 1994).

Drug addiction is a complex and devastating disease. It incorporates genetic and environmental factors that when combined, can hijack neural pathways involved in normal reward memory processing, increase an individual's propensity to abuse drugs, and severely compromise their ability to stop. Fundamentally, drug addiction can be seen as an aberrant learning disorder, as it shares common mechanisms seen in memory acquisition and maintenance (Everitt *et al*, 2001; Hyman *et al*, 2006; Kelley, 2004; Thomas *et al*, 2008; Torregrossa *et al*, 2011). However, while our knowledge of the epigenetic mechanisms governing learning and memory has greatly increased over the last decade, we are only beginning to understand the epigenetic mechanisms underlying drug addiction.

Histone modifications in drug reward

The term 'addiction' refers to a confluence of genetic, physiological, and environmental factors that lead to uncontrolled drug intake and significant lifestyle disruption; however, current behavioral models of addiction focus primarily on the rewarding and motivational aspects of drug intake. More specifically, animal models in the field of addiction epigenetics primarily focus on behavioral responses to administration of drugs of abuse as a way to measure drug reward. While more comprehensive behavioral models would benefit the interpretation of epigenetic studies, assessment of drug-induced molecular changes and how they contribute to the behavior changes are critical for understanding the development of addiction.

Histone acetylation Most of our knowledge on epigenetic regulation of addiction has focused on the effects of psychostimulants such as cocaine and amphetamine on histone modifications. Among these, the most common modifications studied involve histone acetylation and methylation. Cocaine exposure alters acetylated H3 and H4 levels in the NAc (Kumar *et al*, 2005; Schroeder *et al*, 2008; Shen *et al*, 2008). For instance, acute cocaine exposure increases H4 acetylation at the promoter of *c-Fos*, an immediate early gene and a marker of neuronal activation, while chronic exposure results in no such a change (Kumar *et al*, 2005; Renthall *et al*, 2008). Nevertheless, chronic cocaine exposure can also result in gene activation that is not induced by acute treatment. One such example is the acetylation of H3 at the *BDNF* and *CDK5* promoter regions (Kumar *et al*, 2005). While cocaine administration can alter histone acetylation at many gene promoters, it does not necessarily result in altered transcription in the NAc (Renthall *et al*, 2009). It is worth mentioning that the lack of correlation does not imply that a similar changes in *BDNF* promoter acetylation have been detected following cocaine exposure (Sadri-Vakili *et al*, 2010), but rather highlights the complexity of transcriptional output resulting from changes in histone acetylation.

Consistent with the above studies, behavioral tests measuring the effect of HDAC deletion on cocaine sensitivity and reward have also resulted in mixed outcomes. For instance, while deletion of *HDAC1* in the NAc attenuates behavioral responses to cocaine, deletion of *HDAC2* or *HDAC3* in the NAc does not (Kennedy *et al*, 2013). Interestingly, inhibition of HDAC3, the most highly expressed HDAC in the brain (Broide *et al*, 2007), enhances extinction and prevents reinstatement of cocaine seeking in a conditioned place preference paradigm (Malvaez *et al*, 2013). To date, most behavioral studies investigate the effects of psychostimulants on drug seeking and locomotor sensitization. However, to obtain a more complete picture on the role of epigenetic modifications in drug addiction, behavioral models of addiction more similar to the human condition, such as intravenous drug self-administration, should be considered.

Histone methylation Several recent studies have investigated the effects of drugs of abuse on histone methylation states. While drug exposure fails to have a general effect on HMTs and HDMs, chronic cocaine treatment represses G9a in the nucleus accumbens, as evidenced by decreases in H3K9 dimethylation (Maze *et al*, 2010). Additionally, G9a inhibition in NAc, either genetically or pharmacologically, increases behavioral responses to cocaine and opiates, and overexpressing G9a can reverse these effects (Maze *et al*, 2010; Sun *et al*, 2012). Furthermore, Cre-dependent knockout of G9a in the NAc increases dendritic arborization (Maze *et al*, 2010), suggesting

that H3K9 dimethylation by G9a may play a role in drug-dependent synaptic plasticity. Mechanistically, G9a appears to play a central role in a negative feedback loop with Δ FosB, a long-lasting transcription factor central to drug addiction (Feng & Nestler, 2013; Robison & Nestler, 2011). G9a inhibits induction of Δ FosB, and in turn, Δ FosB inhibits expression of G9a (Maze *et al.*, 2010; Sun *et al.*, 2012). Additionally, prolonged HDAC inhibition not only inhibits behavioral responses to cocaine, but also induces G9a expression, a finding consistent with the ability of G9a overexpression to inhibit such behavioral responses to psychostimulants (Kennedy *et al.*, 2013).

While these findings support the involvement of epigenetic regulation in drug reward, one also cannot undermine the role of transcription factors in the recruitment and modulation of epigenetic modifying enzymes. Indeed, transcription factors such as Δ FosB, myocyte enhancer factor 2 (MEF2), and CREB are all known to recruit epigenetic modifying enzymes (Robison & Nestler, 2011). Δ FosB can drive CDK5 transcription by recruiting CBP (Levine *et al.*, 2005) and, conversely, inhibit c-Fos transcription by recruiting HDAC1 (Renthal *et al.*, 2008). MEF2 can recruit the class II HDAC, p300, while CREB also binds CBP (He *et al.*, 2011a; Robison & Nestler, 2011). It is therefore likely that transcription factors and epigenetic enzymes work in concert to mediate the transcriptional regulation of drug reward.

DNA methylation in drug reward

There are relatively few studies to date that focus on the role of DNA methylation in drug reward and addiction. It is known that acute and chronic cocaine exposure promotes Dnmt3a expression in the NAc (Anier *et al.*, 2010; LaPlant *et al.*, 2010). More specifically, 28-day cocaine withdrawal increases Dnmt3a levels in the NAc regardless of whether the cocaine is self-administered or delivered in a non-contingent manner. With regard to a causal relationship, inhibition of Dnmt3a in the NAc via knockdown or via pharmacological administration of RG108 increases behavioral responses to cocaine. Conversely, overexpression of Dnmt3a shows the opposite, blunting cocaine reward. Plasticity is also affected, as chronic cocaine use increases accumbal thin dendritic spine density, an effect mimicked by local overexpression of Dnmt3a (LaPlant *et al.*, 2010). In addition to Dnmt3a, the methyl-CpG binding protein MeCP2 has also been linked to addiction. MeCP2 contributes to gene silencing by recruiting HDACs to methylated DNA (Amir *et al.*, 1999; Van Esch *et al.*, 2005). Chronic cocaine self-administration in rats increases striatal MeCP2 levels. Interestingly, when MeCP2 is locally knocked down in the striatum, rats decrease their cocaine intake levels (Im *et al.*, 2010). Conversely, genetic ablation of MeCP2 in the NAc enhances amphetamine reward (Deng *et al.*, 2010). While Dnmt3a and MeCP2 appear to regulate aspects of drug reward, no direct evidence of differential methylation of addiction-related genes has been shown. Regardless, available evidence suggests a cocaine reward-blunting role for Dnmt3a in the NAc and, in the case of MeCP2, a paradoxical pattern of epigenetic regulation of drug reward that is anatomically discrete.

Until recently, DNA demethylation has been a topic of much speculation. It is clear now that TET enzymes regulate DNA demethylation and likely play a central role in learning and memory (Guo *et al.*, 2011a,b; Kaas *et al.*, 2013; Ma *et al.*, 2009; Rudenko *et al.*, 2013; Zhang *et al.*, 2013). Similarly, DNA demethylation is likely involved in adaptive and maladaptive changes in gene expression

that contribute to the addiction phenotype. As mentioned earlier, 5hmC is a DNA demethylation intermediate highly enriched in the brain, and intragenic 5hmC is associated with gene transcription (Szulwach *et al.*, 2011). To date, studies that aim to profile genomic methylation states utilize bisulfite sequencing that fails to distinguish 5mC from 5hmC. This limitation disguises genomic regions of active demethylation in favor of transcriptionally repressive methylated DNA. However, TET-assisted bisulfite sequencing (TAB-Seq) allows for 5hmC detection at single-base resolution (Yu *et al.*, 2012) and may provide a useful tool to more adequately investigate the effects of drugs of abuse on brain DNA methylation states.

MicroRNAs in drug reward

Recent studies suggest that some of the epigenetic events can be mediated by microRNAs (Bali & Kenny, 2013). Interestingly, cocaine exposure can modulate microRNA (miRNA) levels in the NAc, such as upregulation of miR-181 and downregulation of miR-124 and let-7d. Importantly, modulation of miRNA levels corresponding to the changes seen following cocaine exposure can potentiate behavioral responses to the drug (Chandrasekar & Dreyer, 2009, 2011), suggesting that transcriptional regulation of addiction-related genes by miRNA is sufficient to increase susceptibility to drug reward. This also implies that opposite directional manipulation of striatal miRNA can curb cocaine reward and consumption. Indeed, overexpression of striatal miR-212 reduces cocaine intake in rats through increasing activity of CREB, a transcription factor that opposes cocaine reward (Hollander *et al.*, 2010; Robison & Nestler, 2011). These results further suggest that in addition to histone and DNA modifications, miRNAs can also play a significant role in the development of addiction.

Transgenerational inheritance of drug phenotypes

A classical interpretation of an epigenetic change dictates that it must be heritable, and recent evidence suggests that preference for drugs of abuse can also be inherited to subsequent generations. For example, offspring of alcohol-preferring rats, when compared to offspring of non-alcohol-preferring rats, show increased nicotine intake and reinstatement following extinction, yet remarkably, do not show any difference in cocaine intake (Le *et al.*, 2006). In the case of psychostimulants, male offspring from cocaine-experienced sires display a cocaine-resistant phenotype, but normalize intake when BDNF signaling is pharmacologically inhibited (Vassoler *et al.*, 2013). Interestingly, cocaine-experienced sires show increased H3 acetylation and BDNF expression in sperm, indicating germline epigenetic reprogramming (Vassoler *et al.*, 2013). It is also important to note that while the amount of drug consumed is a reliable reflection of the reinforcing properties of a drug, this is only one metric of addiction and does not encompass the complete behavioral spectrum commensurate with drug addiction. Regardless, prolonged drug use shows the potential to promote heritable epigenetic modifications that could place progeny at increased vulnerability for drug abuse later in life.

Open questions and future directions

The examples presented above suggest that epigenetic regulation is part of the mechanism underlying addiction. It is thus possible that epigenetic modifications in dopaminergic VTA neurons by local

TET/TDG-driven demethylation could regulate synaptic plasticity in the hippocampus, promoting consolidation of long-term reward memories in the PFC (Fig 3). Of course, this hypothesis focuses solely on dopaminergic output, discounting other local cell types that may contribute to the phenotype. As such, one area in the study of epigenetic mechanisms of addiction that remains largely unaddressed is the neurochemical resolution at which epigenetic changes occur to effect behavioral changes. Most of the studies presented in this review focus on individual brain regions and not cell types. Just as there are transcriptional repressors and silencing markers that maintain gene expression under control, heterogeneity of neuron types within a given brain region may have a role in regulating neuronal activity. For instance, in addition to dopamine neurons, the VTA is also populated by GABA neurons. Indeed, activation of VTA GABA neurons is known to suppress excitation of dopamine neurons (van Zessen *et al*, 2012), yet repeated exposure to cocaine has been shown to disinhibit dopamine neurons via reduced activity of GABA neurons within the VTA (Bocklisch *et al*, 2013). This suggests that we cannot make absolute claims about the role of epigenetic mechanisms on behavior based solely on a single brain region unless we are able to precisely select a homogeneous neuronal population and focus our manipulations and analysis on said group of cells. Furthermore, as discussed earlier, caution should be taken when attributing findings from pharmacological studies on epigenetic modifying enzymes to addiction and learning behavior as these may lack target specificity or be ineffective in postmitotic neurons. However, recent advances in developing genome-editing tools will soon allow us to investigate the role of specific epigenetic modifying enzymes in the development of the addictive process within distinct neuronal populations. For instance, the CRISPR/Cas9 system can facilitate this process by excising multiple genetic targets with remarkable precision (Cong *et al*, 2013; Ran *et al*, 2013a,b). This technology may permit the study of various epigenetic modifying enzymes within specific subsets of neurons *in vivo*, thus addressing a major challenge in the field. Additionally, some of the studies discussed in this review correlate subtle changes in epigenetic states with distinct behavioral phenotypes without addressing the cause-and-effect relationship. In this regard, CRISPR/Cas9 technology will be very useful in addressing this issue as it may allow precise manipulation of epigenetic states at specific genomic loci.

As mentioned earlier in this review, the study of addiction epigenetics could benefit from behavioral models that more accurately mirror drug intake in humans, such as intravenous drug self-administration. This behavioral paradigm assesses the reinforcing properties of a drug, where animals perform a learned operant task (lever pressing) in order to receive an intravenous infusion of a drug (Fowler & Kenny, 2011; Tuesta *et al*, 2011). This is a particularly important distinction because the experimental animal has absolute control over its drug intake, as opposed to reacting to a non-contingent drug challenge that can result in altered stress hormone transmission in the brain (Palamarchouk *et al*, 2009). While technically challenging, drug self-administration in mice yields valuable insights into acquisition behavior, compulsivity, and relapse to drug seeking (Fowler & Kenny, 2011). Thus, it can provide a more complete behavioral model of drug addiction, especially given that a majority of genetic manipulations are currently performed in mice.

There are relatively few studies that focus on the role of DNA methylation and drug addiction, and to date, there are no studies

that look at the role of DNA demethylation. The process of DNA methylation has long been considered to be a stable, static process, but with our recent understanding of the molecular mechanisms of DNA demethylation catalyzed by the TET and TDG enzymes, DNA methylation appears not to be as stable as previously thought. It will therefore be necessary to address the specific role that DNA demethylation machinery plays in the addiction process. Despite the challenges, regulation of DNA methylation states has the potential to serve as a molecular switch that can drive memory formation and shape vulnerability to substance abuse disorders.

The role of epigenetic mechanisms in learning and memory still remains a nascent field of study; yet accumulating evidence suggests that epigenetic mechanisms can regulate the ability to store long-term memories. Maintenance of these memories can last for the lifetime of an individual and it is intriguing to speculate how drugs of abuse can potentially induce similar lasting changes in reward pathways that may predispose a person to addiction. Drug addiction is an exciting new frontier for investigation, as it is a behavior affected by numerous genetic and environmental factors. The multifactorial nature of the disease requires interdisciplinary contributions. In order to develop a molecular understanding of addiction, we will need to use genomic tools, such as RNA-Seq, whole-genome bisulfite sequencing (WGBS), and Tet-assisted bisulfite sequencing (TAB-Seq) to determine the effects of drugs of abuse on global epigenetic modifications and gene expression. When combined with powerful behavioral techniques such as intravenous self-administration throughout various stages of the addictive process and cell-specific manipulation of gene expression, our understanding of epigenetic mechanisms of addiction may yield exciting new avenues for therapeutic intervention.

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Conflict of interest

The authors declare that they have no conflict of interest.

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