Drug Addiction and DNA Modifications

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Abstract

Drug addiction is a complex disorder which can be influenced by both genetic and environmental factors. Research has shown that epigenetic modifications can translate environmental signals into changes in gene expression, suggesting that epigenetic changes may underlie the causes and possibly treatment of substance use disorders. This chapter will focus on epigenetic modifications to DNA, which include DNA methylation and several recently defined additional DNA epigenetic changes. We will discuss the functions of DNA modifications and methods for detecting them, followed by a description of the research investigating the function and consequences of drug-induced changes in DNA methylation patterns. Understanding these epigenetic changes may provide us translational tools for the diagnosis and treatment of addiction in the future.

Keywords

drug addiction • DNA methylation • DNA modification • DNMT • TET • cocaine • alcohol • nicotine

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6.1 DNA Epigenetic Modifications

The nucleus of a mammalian cell houses approximately 2 m of negatively charged DNA. In order to package such a large amount of genetic material into a nucleus measuring ~10 μm across, multiple means of compaction are required. DNA is tightly wrapped around positively charged histone proteins to form the nucleosome, the founding unit of the DNA packaging material called chromatin [1]. The DNA and histone proteins can be chemically modified in numerous ways in order to change the binding relationship of the DNA-nucleosome complex. Tightly bound nucleosomal DNA is considered heterochromatin, which is generally transcriptionally inactive due to restricted access to DNA by transcriptional machinery. Loosely bound or nucleosome-free DNA is considered euchromatin, which is freely accessible to the transcriptional machinery and actively transcribed [2]. DNA itself can be covalently modified by a class of enzymes called DNA methyltransferases (DNMTs) which catalyze a reaction that adds a methyl group to the C5 position of a cytosine base (5mC) and is traditionally observed at cytosine-guanine dinucleotide (CpG) residues [3]. Replicative maintenance of DNA methylation, copying an existing 5mC onto the complementary DNA strand, following cell division, is accomplished by the maintenance DNA methyltransferase DNMT1 [4]. DNMT3a and DNMT3b are considered de novo methyltransferases, responsible for methylating previously unmethylated cytosines to establish a pattern of DNA methylation [5, 6].

Until recently, it wasn't known if or how DNA methylation was reversed. Several proteins (GADD45a, MBD2, DNMT3a, and DNMT3b) have been reported to catalyze DNA methylation, either by direct removal of methyl groups or via oxidation and repair by DNA repair processes. However, subsequent reports failed to substantiate these claims [7]. In 2009, it was demonstrated that the TET family of proteins (ten-eleven translocation proteins) oxidizes 5mC to 5-hydroxymethylcytosine (5hmC) [8, 9]. 5hmC can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine, which can then be recognized by DNA damage pathways and repaired to unmethylated cytosine [10–15]. TET-mediated oxidation of mC to 5hmC can be an active process in the brain [16]. Interestingly, 5hmC is relatively stable [17] and present at higher levels in the brain than any other tissue [18], suggesting a specific function for 5hmC in the genetic regulation of neuronal function. Thus, we now recognize that DNA epigenetic modifications can be a labile mechanism for regulation of gene expression in non-mitotic neuronal populations (Fig. 6.1).

DNA methylation can have differential effects on transcriptional capacity, depending on the genomic context. 5mC within gene promoter regions is typically associated with a decrease in transcription, and these effects have been well studied. The presence of 5mC attracts methyl-binding domain proteins (MBD1-MBD4, MeCP2, and Kaiso) which, in turn, recruit repressor complexes [3] and histone deacetylases (HDACs) [19] to downregulate local transcriptional activity. Deacetylation of histone tails increases the affinity of the DNA-nucleosome interaction, thereby generating local regions of heterochromatin and decreased transcriptional capacity for the region. Extensive DNA methylation can result in complete

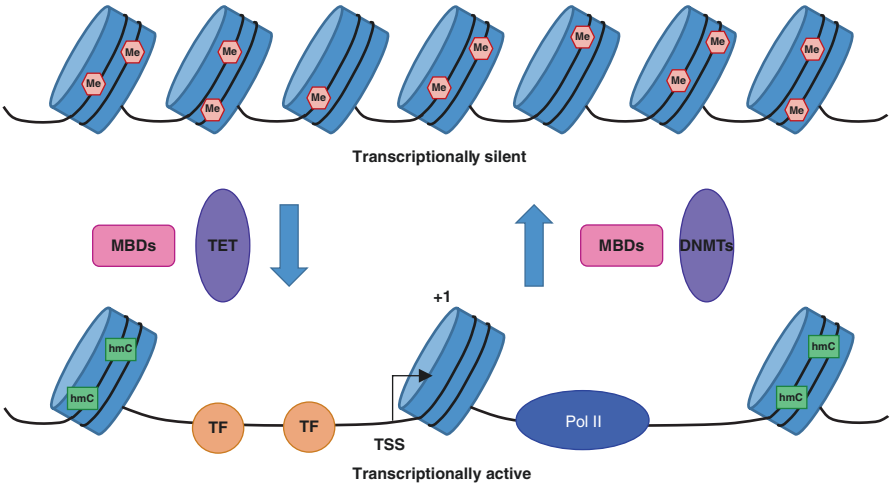

Fig. 6.1 DNA methylation and transcriptional activity (attached). When DNA is heavily methylated (*top*) by DNA methyl transferases (DNMTs), chromatin is tightly packaged and bound by methyl-CpG-binding domain (MBD) proteins, which recruit other heterochromatinizing proteins and render genic regions inaccessible and transcriptionally inactive. Genes lacking 5-methylcytosine (5mC) marks are more loosely packaged into chromatin, readily accessible by the transcriptional machinery and more likely to be transcriptionally active. TET proteins can oxidize 5-methylcytosine to 5-hydroxymethylcytosine (5hmC), which can also bind MBDs. However, in contrast to 5mC, 5hmC within enhancer and genic regions is associated with transcriptional activity

silencing of a gene or local cluster of genes, as has been shown during neuronal fate specification/development [20]. DNA methylation can also interfere with the specific binding of transcription factors, which can only bind to an unmethylated version of its binding site. 5mC within a gene body has been linked to active transcription [21], transcriptional elongation [22] and alternative splicing [23].

The functional consequences of 5hmC are just recently being recognized, but seem to be independent from those of 5mC. The mark is enriched within transcriptionally active genes, enhancers, and brain MBD proteins like MeCP2 which binds the mark with similar affinity as to 5mC [24, 25]. In fact, using mouse embryonic stem cells in a screen for CpG-binding proteins, researchers found few proteins bound preferentially to 5hmC. 5fC, however, was enriched for specific protein binding of several chromatin remodeling proteins and transcriptional regulators [26]. Whether 5hmC or even 5fC enrichment or depletion is correlated with transcription levels has yet to be determined, as results have varied depending on the model system and genomic context under investigation [14]. For example, the genomic distribution of 5hmC differs between neurons and embryonic stem cells. In neurons, 5hmC is enriched in gene bodies of expressed genes related to neuronal function [24], while in embryonic stem cells 5hmC is enriched at enhancers and depleted from transcription factor binding sites [27].

6.2 Addiction

Addiction is a relapsing neuropsychiatric disorder characterized by compulsive drug seeking with repeated and increased use, despite adverse consequences. Drugs of abuse include, but are not limited to, cocaine, nicotine, amphetamine, methamphetamine, heroin, morphine, and other opiates. Addictive drugs stimulate the brain's natural reward system through the release or synaptic accumulation of the neurotransmitter dopamine ([28, 29]). Stimulation of the reward system also engages learning responses in the brain. With repeated drug use, the dopamine-producing cells increasingly respond to drug-associated cues—environmental stimuli commonly experienced with drug use (people, places, smells, imagery)—such that the cues alone elicit a dopamine response and drive craving for the drug [30]. Whereas natural rewards would normally cause dopamine cells to stop firing once a reward is achieved, drugs of abuse override this process and continue to stimulate powerfully high amounts of dopamine release. The excessively rewarding effects of drugs often override the more balanced dopamine released by natural rewards. Eventually, natural rewards become less reinforcing, and motivation switches to achieving the elevated dopamine release generated by the drugs. As the brain adapts to elevated dopamine levels, tolerance to the drug begins to develop, wherein increasing amounts of the substance are required for the user to achieve the desired degree of euphoria. However, in the absence of the drug, the user experiences a hypodopaminergic, dysphoric state and may seek out the drug just to relieve the discomfort. Thus, addiction becomes a vicious cycle in which the user seeks to relieve the symptoms of the disease by engaging in the behaviors which initiated the disease to begin with [29, 31]. Addiction is a worldwide problem which significantly impacts the health, economic, and social fabric of billions of people. In order to relieve this burden, researchers have sought to understand the genetic and environmental causes of substance use disorders.

Addiction is a complex disease resulting from a combination of both genetic and environmental risk factors. It is estimated that only about 10% of people exposed to addictive drugs will experience a severe substance use disorder [32], while the remaining 90% have protective genetic and/or environmental factors. In order to better understand the genetic factors involved in addiction, human studies have been conducted in drug addicts, former drug addicts, and postmortem brains of addicts. Many such studies have identified associations between drug use and allelic variants which may predispose an individual to risk-taking or drug-seeking behaviors. These genes are often related to neurotransmitter function or synaptic plasticity and include serotonin transporter and receptors, dopamine transporter and receptors, opioid receptors, GABA receptors, and MAOA (reviewed by [33]).

6.3 Neuroepigenetics of Addiction

In neuroscience, epigenetic studies have begun to help explain how a genetically stable, nondividing population of neurons can make activity-dependent changes in gene expression of either transient or lasting duration. Changes in DNA methylation

around synaptic plasticity genes of neurons and nonneuronal cell types of the brain accompany the acquisition and maintenance of memory [34, 35] and changes in hydroxymethylation levels correlated with transcriptional and behavioral outcomes have been identified following fear extinction [36] and stress [37]. The DNA methylation detection and quantitation methods commonly used in neuroepigenetic studies have recently been applied to the study of addiction. While there have been several candidate gene studies of DNA methylation changes following drugs of abuse (detailed below), few have explored genome-wide changes in DNA methylation (Table 6.1). High-throughput sequencing of DNA methylation analyses can provide a global view of such changes with a potential benefit at single-base pair resolution and, coupled with mRNA sequencing transcriptome profiling, can help researchers probe the associations between changes in DNA methylation and transcriptional outcomes observed in addiction models.

6.3.1 Human Studies

Several human epigenome-wide association studies (EWAS) have linked genome-wide DNA methylation changes in whole blood samples to cigarette smoking (reviewed by [69]). From these EWAS studies and locus-specific methylation studies, several candidate genes have been identified as harboring DNA methylation changes among cells isolated from smokers' blood samples: MAOA (monoamine oxidase A) [40], MAOB (monoamine oxidase B) [64], COMT (catechol o-methyltransferase) [65, 68], AHRR (aryl-hydrocarbon receptor repressor) [67], and POMC (proopiomelanocortin) [66]. Cigarette smoking has also been linked to changes in DNA methylation in several tissue and cell types; however, non-nicotinic chemicals present in cigarettes can also lead to DNA damage and changes in DNA methylation and gene expression related to inflammation or hypoxia [70–72], making analysis of the effects of cigarette smoking a complicated endeavor.

Alcohol dependence has also been associated with genome-wide changes in blood cell DNA methylation [73, 74], some of which have been shown to reverse with the progression of abstinence [48]. Gene-specific studies have also shown an association between alcohol dependence and hypermethylation of the DAT (dopamine transporter) promoter, HERP (homocysteine-induced endoplasmic reticulum protein) promoter, and α-synuclein promoter [38, 39, 42], while POMC promoter methylation has been linked to alcohol dependence [46] and craving in alcohol-dependent subjects [43]. In addition, the severity of alcoholics' drinking patterns was found to be negatively correlated to DNA methylation of a cluster of CpGs associated with the promoter region of the NR2B (NMDA receptor 2B) gene [41]. Using postmortem human brains, researchers found an association between alcohol dependence and differential DNA methylation within the 3′-UTR of the PDNY (prodynorphin) gene [44] as well as hypomethylation of endogenous retroviruses in the frontal cortex of alcoholics [45].

CpG sites within the BDNF (brain-derived neurotrophic factor) promoter of patient blood cells have been shown to be significantly associated with

Table 6.1 Alterations of DNA epigenetic modifications in addiction

Drug of abuse	Genomic region	Gene(s)	Direction of change	Associated mRNA/protein change	Species	Tissue/cell type	DNA methylation method	References
Alcohol	Promoter	α-Synuclein	Increased DNA methylation	Elevated homocysteine levels	Human	Whole blood	Restriction endonuclease/qPCR	[38]
Alcohol	Promoter	HERP	Increased DNA methylation	Increased HERP mRNA	Human	Whole blood	Restriction endonuclease/qPCR	[39]
Alcohol	Promoter	MAOA	Increased DNA methylation	N/A	Human women	Lymphoblast	Bisulfite/mass spectrometry	[40]
Alcohol	Promoter	NR2B	Decreased DNA methylation	Increased NR2B mRNA	Human	Peripheral blood cells	Bisulfite/sequencing	[41]
Alcohol	Promoter	DAT	Increased DNA methylation	N/A	Human	Leukocytes	Restriction endonuclease/qPCR	[42]
Alcohol	Promoter	POMC	Increased DNA methylation	N/A	Human	Whole blood	Bisulfite/sequencing	[43]
Alcohol	3′-UTR	PDNY	Increased DNA methylation	Increased PDNY mRNA	Human	Postmortem PFC	Bisulfite/sequencing	[44]
Alcohol	Genome-wide	Genome-wide	Decreased DNA methylation	Increased expression of ERVs and CG-rich genes	Human	Postmortem cortex	Microarray	[45]

Alcohol	Genome-wide	GABRB3, POMC, HTR3A, NCAM1, DRD4, MBD3, HTR2B, GRIN1	Increased DNA methylation	N/A	Human	Peripheral blood	Microarray	[46]
Alcohol	Genome-wide	Genome-wide	Decreased DNA methylation	N/A	Human	Lymphocytes	Microarray	[47]
Alcohol	Genome-wide	Genome-wide	Both—diminish with progression of abstinence	N/A	Human	Peripheral blood mononuclear cells	Microarray	[48]
Alcohol	Genome-wide	SYT2	Increased DNA methylation	Decreased mRNA for SYT2 and genes encoding proteins involved in neurotransmitter release	Rats	mPFC	Antibody affinity	[49]
Cocaine	Promoter	PP1c	Increased DNA methylation	Decreased PP1c mRNA	Mouse	NAc	Me-DIP/PCR	[50]
Cocaine	Promoter	CDKL5	Increased DNA methylation	Decreased Cdkl5 mRNA	Rats	Striatum	Bisulfite/qPCR	[51]

(continued)

Table 6.1 (continued)

Drug of abuse	Genomic region	Gene(s)	Direction of change	Associated mRNA/protein change	Species	Tissue/cell type	DNA methylation method	References
Cocaine	Genome-wide	Genome-wide	Increased DNA methylation	N/A	Mouse	NAc	Antibody affinity	[52]
Cocaine	Genome-wide	Genome-wide	Decreased DNA methylation	N/A	Mouse	PFC	LC-ESI-MS/MS	[53]
Cocaine	Promoters	Cocaine-responsive genes	Both	mRNA inversely correlated to DNA methylation changes in cocaine-responsive genes	Mouse	NAc	Me-DIP	[54]
Cocaine	Genome-wide	Genome-wide	Both	N/A	Rats	PFC	MBD Ultra-Seq	[55]
Cocaine	Genome-wide	Genome-wide	Both	Altered expression of alternate splicing isoforms	Mouse	NAc	Bisulfite/oxidative bisulfite/sequencing	[56]
Cocaine	Genome-wide	Genome-wide	Both	mRNA partly inversely correlated to DNA methylation changes	Rats	NAc	Microarray	[57]

Cocaine	Genome-wide	c-Fos	Decreased DNA methylation	Increased c-FOS mRNA	Rats	NAc	LC-ESI-MS/MS	[58]
Heroin/methadone	Promoter	OPRM1	Increased DNA methylation	N/A	Human	Lymphocytes	Bisulfite/sequencing	[59]
Heroin	Promoter	BDNF	Decreased DNA methylation	N/A	Human	Whole blood	Bisulfite/sequencing	[60]
Methamphetamine	Genome-wide	GLUA1, GLUA2	Decreased DNA methylation 5hmC	Decreased GLUA1, GLUA2 mRNA	Rats	NAc	5hmC microarray	[61]
Methamphetamine	Promoter and intronic	Immediate early genes	Both	Decreased mRNA	Mouse	PFC, hippocampus	Bisulfite/sequencing	[62]
Methamphetamine	Genome-wide	KCNMA1, KCNN1, KCNN2	Increased 5hmC	Increased mRNA for KCNMA1, KCNN1, KCNN2	Rats	NAc	hMe-DIP/sequencing	[63]
Methamphetamine	Promoter	BDNF	Decreased DNA methylation	N/A	Human	Whole blood	Bisulfite/sequencing	[60]
Nicotine	Promoter	MAOA	Increased DNA methylation	N/A	Human women	Lymphoblast	Bisulfite/mass spectrometry	[40]

(continued)

Table 6.1 (continued)

Drug of abuse	Genomic region	Gene(s)	Direction of change	Associated mRNA/protein change	Species	Tissue/cell type	DNA methylation method	References
Nicotine	Promoter	MAOB	Decreased DNA methylation	Decreased MAOA protein	Human	Platelet and plasma	Bisulfite/sequencing	[64]
Nicotine	promoter	COMT	Increased DNA methylation	N/A	Human	Whole blood	Bisulfite/sequencing	[65]
Nicotine	Promoter	POMC	Decreased DNA methylation	N/A	Human	Peripheral blood mononuclear cells	Bisulfite/sequencing	[66]
Nicotine	Genome-wide	AHRR	Decreased DNA methylation	N/A	Human	Whole blood	Microarray	[67]
Nicotine	Promoter	COMT	Increased DNA methylation	N/A	Human adolescents	Whole blood	Bisulfite/mass spectrometry	[68]

methamphetamine and heroin addiction [60], and methadone-maintained former heroin addicts have increased DNA methylation at the OPRM1 (opioid receptor mu 1) promoter, leading to a decrease in OPRM1 gene expression in lymphocytes [59]. Exposure to social stressors can even lead to addiction-related changes in DNA methylation patterns. One group showed that lower socioeconomic status during adolescence is associated with increased blood cell DNA methylation in the promoter of the serotonin transporter gene, predicting changes in risk-related brain functions and predisposing these individuals to an increased addiction susceptibility [75].

6.3.2 Animal Studies

While human studies can provide insight into some of the genes involved in the process of addiction, controlled animal studies are necessary to fully investigate and manipulate experimental conditions to display detailed underpinnings. To date, much of the research on addiction has utilized rodent models of exposure. One animal model to human addiction is the self-administration (SA) model, wherein a rodent is trained to press a lever or a button to receive an intravenous infusion of a drug. This model best recapitulates the addiction process, as the animals will seek out the drug more frequently and persistently. Given the cost, time, and technical challenges related to the SA model, many researchers apply intraperitoneal (i.p.) drug injections, and although this model may not engage the brain regions involved in the choices an addict makes, it can successfully elucidate the direct behavioral, chemical, and genetic effects of the drug. Investigators using i.p. drug administration also employ a behavioral conditioning paradigm called conditioned place preference (CPP) to assess an animal's preference for a drug based on their preference to be in the same context or environment as where the drug was administered. These models are considered the standards in addiction research today, and their utilization makes for a more translational approach to understand the disease.

In the 2000s, epigenetic studies of psychostimulant exposure provided a hint that changes in DNA methylation may be occurring. In 2006 it was reported that following 10 days of i.p. cocaine injections, methyl-binding proteins MeCP2 and MBD1 were significantly induced in the caudate-putamen, frontal cortex, and dentate gyrus of adult rats. These changes were accompanied by an increase in HDAC2 (histone deacetylase 2) and deacetylated histones, presumably leading to reduced transcription [76]. It was subsequently shown that cocaine-induced MeCP2 was accompanied by increased MeCP2 binding at the Cdkl5 promoter and repression of the Cdkl5 gene in the striatum of cocaine-treated rats. In order to examine DNA methylation changes, DNA was subjected to sodium bisulfite treatment. Using this method, only unmethylated cytosines are converted to uracil. Subsequent comparison of untreated and bisulfite treated DNA can reveal which cytosines are methylated or unmethylated at single-base pair resolution [77] either at the single-locus level or genome-wide. Using bisulfite-converted DNA and Cdkl5-specific primers, it was shown that DNA methylation at the Cdkl5 promoter was inversely correlated

with transcription of Cdkl5 mRNA [51]. Cdkl5, like MeCP2, is mutated in some forms of the autism-like Rett Syndrome [78]. However, its role in the action of cocaine is still unknown. Similar results were observed studying rats self-administering cocaine; MeCP2 expression was increased in multiple brain reward regions, and knockdown of MeCP2 or pharmacologically inhibiting DNMTs with trichostatin A (a histone deacetylase inhibitor known to induce DNA demethylation [79]) attenuated cocaine self-administration [80–82] and amphetamine reward [83].

In 2010, two papers provided thorough investigations into the complex interactions of MeCP2, BDNF, and a specific microRNA, miR-212. Using a rat self-administration model of cocaine addiction, first, it was shown that expression of miR-212 is increased in the dorsal striatum of rats with extended access to cocaine self-administration and that miR-212 expression was inversely correlated with cocaine intake [84]. However, miR-212 is located in a genomic region dense in CpG islands and may be subject to regulation by MeCP2. Therefore, researchers investigated the interaction between MeCP2, miR-212, and cocaine intake in the same rat self-administration model. They found that miR-212 and MeCP2 expression are inversely correlated with one another; knockdown of MeCP2 increases miR-212 expression, and overexpression of miR-212 inhibits MeCP2 expression. MeCP2 is a known regulator of BDNF [85], which is known to promote sensitivity to cocaine [86]. It was also demonstrated that miR-212 also regulates BDNF expression *indirectly* through repression of MeCP2, such that a complicated feedback loop between BDNF, miR-212, and MeCP2 serves to regulate cocaine-taking behavior [81].

As it became recognized that DNA methylation plays a role in addiction, it was further demonstrated that repeated cocaine administration altered DNMT3a transcription (but not DNMT3b) in the mouse NAc [52]. Interestingly, the changes observed were time dependent; DNMT3a was upregulated 4 h after the last cocaine dose, but was subsequently downregulated 24 h later. Following a 28-day period of withdrawal from either i.p. cocaine or SA, DNMT3a was again found to be upregulated. When DNMT3a was overexpressed in the NAc, mice showed a decreased preference for cocaine in the CPP paradigm. These behavioral changes were accompanied by an increase in DNA methylation, as assayed by an ELISA-like colorimetric assay. In this assay, an antibody to 5mC recognizes methylated DNA, and a secondary antibody produces a color which is proportional to the amount of methylated DNA (Epigentek, Farmingdale, NY). Preference for cocaine could be attenuated by pharmacological inhibition using a DNMT inhibitor, RG108 [52]. The persistent induction of DNMT3a after a month of abstinence from cocaine may be of particular relevance to understanding the molecular susceptibility to relapse and warrants further investigation for potential therapeutic interventions.

In contrast to the previous study, another group reported that, when administered acutely, a single 15 mg/kg injection of cocaine was shown to upregulate both DNMT3a and DNMT3b in the mouse NAc [50]. This prompted an investigation of the DNA methylation status of NAc tissue using an immunoprecipitation-based method called Me-DIP (methylated DNA immunoprecipitation). This technique utilizes an antibody to 5mC to isolate methylated DNA from a pool of fragmented DNA [87]. Downstream analyses of Me-DIP fragments can be used for single-locus

PCR, microarray, or sequencing. The authors found that acute and repeated cocaine resulted in DNA hypermethylation and increased MeCP2 binding to the PP1c promoter, resulting in downregulation of the PP1c gene [50], as was seen with Cdkl5 [51]. Pharmacologically blocking DNMT activity decreased cocaine-induced PP1c hypermethylation and gene expression changes while delaying the development of cocaine-induced behavioral sensitization. However, the opposite effect was seen at the immediate early gene, FosB—DNA became hypomethylated and MeCP2 binding was decreased following a single cocaine injection [50]. Therefore, cocaine may not cause global changes in DNA methylation in a nonspecific manner. Rather, specific genes or networks of genes appear to be co-regulated at the level of chromatin following drug exposure. For example, in 2015, two groups found that chronic methamphetamine or alcohol consumption increased DNA methylation at CpG sites in synaptic plasticity-related genes, resulting in downregulation of associated mRNAs in rat frontal cortex [49, 62].

With increasing evidence that DNA methylation plays an important role in the progression of addiction, withdrawal, and relapse, the possibility of using the methyl donor methionine as a therapeutic gained interest. Pretreatment with methionine has been shown to reduce cocaine-conditioned place preference (CPP) in mice [52]. However, it is unknown if these effects were due to a genuine increase in DNA methylation or some other effects of methionine, as the DNA methylation status was not evaluated under these conditions [52].

Another group compared the rewarding effects of cocaine, morphine, and food using the CPP procedure and evaluated resulting changes in global DNA methylation by LC-ESI-MS/MS (liquid chromatography-electrospray ionization tandem mass spectrometry) [53]. In this method, LC is used to separate 5mC from the other nucleotides, and ESI-MS/MS can detect and quantify 5mC with high specificity and sensitivity [88]. This method can provide reliable quantitation of global DNA methylation levels with very low amounts of input DNA, but cannot be used to determine specific methylation patterns. Using this method, researchers found that cocaine, but not food or morphine, decreased DNA methylation and DNMT3b expression in the mouse prefrontal cortex. Treatment with methionine before and during the CPP procedure blocked the cocaine-induced decrease in DNMT3b expression and DNA methylation and attenuated cocaine preference, but had no effects on the establishment of food or morphine preference [53].

Conversely, it was shown that pretreatment of mice with methionine for 7 days significantly potentiated the development of cocaine-induced locomotor sensitization. NAc whole-genome gene expression profiling revealed that repeated SAM treatment affected cocaine-induced gene expression, nonspecifically dampening the cocaine response, in part due to decreased methyltransferase activity via downregulation of *Dnmt3a* mRNA. Using Me-DIP, they found specific hypo- and hypermethylation in the promoters of cocaine-responsive genes in the nucleus accumbens [54].

In 2015, another group similarly examined these changes in the nucleus accumbens of cocaine-sensitized and self-administering rats with or without methionine pretreatment. They showed that methionine pretreatment can upregulate DNMT3a

and DNMT3b, and LC-ESI-MS/MS revealed global DNA hypomethylation in the NAc of cocaine-treated rats. The treatment blocked locomotor sensitization and reduced cocaine-primed reinstatement of self-administration. Conversely, the cocaine-induced upregulation and hypomethylation of c-Fos was reduced in rats receiving methionine, [58] again demonstrating that cocaine-induced changes in DNA methylation (as well as methionine-reversed changes) are likely gene-specific events. While the locomotor-sensitizing effects of methionine differ between the [54] study and the [58] study, this is possibly due to the differing routes of cocaine administration, as experimenter-administered injections do not engage the same circuits in the brain as does the self-administration model. Nevertheless, they show promise for nutritional supplementation with agents like methionine as a potential method of promoting or restoring a healthy methylome.

Not only does the experimental paradigm differentially affect DNA methylation, but abstinence and withdrawal also have characteristic changes in DNA methylation patterns. Using MBD Ultra-Seq, a method in which DNA fragments immunoprecipitated by MBD antibodies are sequenced [36], researchers found that 29 regions of the genome were differentially methylated in the medial prefrontal cortex of cocaine self-administering rats, but not in response to experimenter-administered cocaine. Furthermore, an additional 28 regions became differentially methylated during forced abstinence or withdrawal from cocaine [55]. In a similar study using Me-DIP coupled with a custom tiling microarray, it was found that, in addition to significant DNA methylation changes in the NAc during withdrawal from cocaine self-administration, cue-induced cocaine seeking (a model of a relapse paradigm) caused broad, time-dependent enhancement of DNA methylation alterations which were, in part, negatively correlated to gene expression. In addition, intra-NAc injections of DNMT inhibitor RG108, ESR1 agonist propyl pyrazole triol, and CDK5 inhibitor roscovitine each reduced or completely abolished cue-induced cocaine seeking [57]. These data show that DNA methylation and downstream targets of DNA methylation are viable targets for the treatment of drug craving and addiction.

With the advancement of molecular genetic techniques, researchers are now able to differentiate between different types of DNA methylation, namely, 5mC and 5hmC, which had previously been indistinguishable and lumped together using older methods. In the last few years, 5hmC has become recognized as a functional DNA modification that may lead to DNA demethylation. Using Me-DIP and hMe-DIP (hydroxymethylcytosine DNA immunoprecipitation), researchers showed that chronic methamphetamine treatment decreased enrichment of 5mC and 5hmC at the GluA1 and GluA2 genes while conversely increasing MeCP2 binding and decreasing GluA1 and GluA2 gene expression in rat striatum [61]. In addition, methamphetamine-addicted rats show differential 5hmC patterns in the nucleus accumbens, as determined using hMe-DIP sequencing. These changes were primarily concentrated in intergenic regions. However, differential 5hmC changes within gene bodies correlated with increased transcription of that gene product [63].

The TET1 enzyme, which is responsible for the oxidative conversion of methylated cytosine to hydroxymethylated cytosine, was shown to be downregulated in the nucleus accumbens of mice treated with cocaine as well [56]. This downregulation of TET1 was also found in the same brain region of cocaine addicts, when examined postmortem. Using bisulfite and oxidative bisulfite sequencing, 5hmC was elevated within enhancer and coding regions of the genome. When TET1 function was overexpressed or knocked down, it negatively regulates cocaine reward-type behaviors. Specifically, these intragenic changes in 5hmC increased expression of alternate splicing isoforms of many genes with important roles in addiction and could persist for at least one month following drug exposure [56].

6.4 Multigenerational Effects of Drug Exposure

Recent work has demonstrated that exposure to various chemical and environmental stressors can also cause changes in DNA methylation and transcriptional output, which can be transmitted to subsequent generations. Several groups have shown that parental exposure to drugs of abuse can have significant behavioral, biochemical, and neuroanatomical effects on the offspring (reviewed by [89]). Epigenetic mechanisms, such as DNA methylation and histone modifications, have been attributed to many such effects. For example, children exposed to cigarette smoke in utero also have altered patterns of DNA methylation within repetitive DNA elements LINE1 and AluYb8, which persisted through at least age 6 [90]. Rats exposed to cocaine during prenatal development have altered patterns of hippocampal DNA methylation with corresponding changes in transcriptional output [91].

Drug exposure during embryonic development not only exposes the developing fetus (F1) to the effects of the drug but also exposes the germ cells (F2) to these effects as well. Similarly, parental drug use exposes *their* germ cells, effectively exposing the F1 generation. Adolescent rat exposure to cannabinoid receptor agonist WIN 55,212–2 or THC caused genome-wide changes in male and female F1's DNA methylation status, associated changes in gene expression, and enhanced F1 offspring's sensitivity to morphine [92–95]. Research has revealed that altered patterns of DNA methylation can be transgenerationally inherited *beyond* the exposed generations (F3 for embryonic exposure and F2 for parental exposure) [96, 97]. This was shown for animal models in which the parents were exposed to chemical and environmental stressors such as stress [98], plastics and endocrine disruptors [99–101], pesticides, jet fuel, and dioxin [102, 103]. The epigenetic effects of prenatal exposure to the endocrine disruptor vinclozolin were shown to be transmitted through DNA methylation in the male germ cells [104]. Rodents self-administering cocaine show decreased DNMT1 in the seminiferous tubules [105] and males who consume heavy amount of alcohol have a reduction in hypermethylated, paternally imprinted regions of the sperm genome [106], indicating that cocaine and alcohol may also have DNA methylation effects on the male germ line which could be transmitted to subsequent generations.

Conclusion

The state of neuroepigenetic addiction research has progressed to a point where we can apply cell-specific, high-throughput technologies to determine drug-specific effects on DNA methylation and corresponding transcriptional and behavioral output. Thorough understanding of the mechanisms that drive the addiction process will enable researchers to develop diagnostic biomarkers and better therapeutic strategies for treatment and prevention of substance use disorders. As demonstrated with the transgenerational studies, efforts toward combating drug use and addiction will contribute to furthering the health and fitness of the worldwide population for generations to come.

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