Chapter 2

Epigenetics and Drug Abuse

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Additional information is available at the end of the chapter

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Abstract

Gene expression and inheritance are not only a function of the DNA code, but also epigenetic mechanisms that regulate DNA accessibility, transcription, and translation of the genetic code into a functional protein. Epigenetic mechanisms are invoked by life experiences, including stress and exposure to drugs of abuse, and the resulting changes in gene expression can be inherited by future generations. This chapter highlights recent research demonstrating epigenetic changes in response to drug exposure with a focus on three different mechanisms: DNA methylation, histone modification, and noncoding RNAs. We briefly describe each of these mechanisms and then provide key examples of drug-induced changes involving these mechanisms, as well as epigenetic manipulations that alter effects of drugs. We then review cutting-edge technologies, including viral-mediated gene transfer and gene editing, that are being used to manipulate epigenetic processes with temporal and cell-type specificity. We also describe and provide examples of intergenerational epigenetic modifications, a topic that has interesting implications for how addiction-related traits may be passed down across generations. Finally, we discuss how this research provides a greater understanding of drug addiction and may lead to novel molecular targets for preventions and interventions for drug abuse.

Keywords: DNA methylation, histone modification, noncoding RNA, cocaine, alcohol

1. Introduction

One of the most compelling questions in the field of drug abuse is why some individuals who experiment with drugs go on to develop substance use disorders (SUDs) while others do not. Both a family history of SUDs and stressful life events increase one's vulnerability to develop SUDs [1, 2]. Historically, these risk factors were viewed as "nature and nurture" making separate contributions to an addiction phenotype. However, recent advances in the field of epigenetics

demonstrate that "nurture" changes "nature" by modifying whether or not a given gene will be expressed. Understanding how one's environment (e.g., drug-taking behavior, stress, and learning) can alter gene expression in the brain may give insight into how drug addiction develops, how it may be passed down into future generations, and perhaps, how it can be better treated.

While the DNA sequence of a gene can be modified directly (e.g., mutations, deletions, insertions, translocations, etc.) resulting in altered gene expression, epigenetics regulates gene expression by mechanisms other than changes to the DNA sequence. It has long been known that epigenetic mechanisms largely control cell differentiation by allowing some genes to be expressed and others to be silenced at various points in time during development. Indeed, even though all human cells possess the same DNA (with the exception of egg and sperm cells), what differentiates a given cell type from others (e.g., a neuron versus a liver cell) is the epigenetic mechanisms that permit or deny its genes to be transcribed and translated into cell type-specific functional proteins [3]. Beyond the hard-wire epigenetic programming of gene expression during development, epigenetic mechanisms also provide dynamic and heritable means of altering gene expression in response to environmental change. For example, either stressful life experiences or a history of chronic drug intake can invoke chemical modifications to either the DNA or the histone proteins that are involved in storing the DNA. Such epigenetic changes have an impact on how accessible the DNA is for gene transcription. Epigenetic changes can also be long lasting and passed down to future generations. In this way, not only does experience with stress and/or drugs place one's self at risk for SUDs, but also one's offspring due to heritable epigenetic modifications. Even in the more proximal time frame of an individual's lifespan, epigenetic mechanisms provide a "working memory" for gene expression changes that are involved in brain plasticity [4]. Brain plasticity changes resulting from drug exposure are thought to be the crux of the dysfunction underlying addiction [5]. An exciting implication of understanding the role of epigenetic changes in drug-induced brain plasticity is that new strategies for therapeutic interventions may be discovered.

In this chapter, we review three epigenetic mechanisms that have been found to impact drug abuse-related behaviors in animal models: (1) chemical modifications to DNA, (2) chemical modifications to histones, and (3) the induction of noncoding RNAs that regulate gene expression. We will begin with a brief explanation of how drugs modify intracellular signaling pathways that propagate to the cell nucleus, leading to epigenetic changes. We will then provide a brief description of the epigenetic mechanisms listed above, followed by examples of how drugs of abuse invoke these mechanisms and how pharmacologically targeting the epigenome can alter drug-abuse-related behavior. Next, we will cover the latest developments in genetic tools that provide precise manipulation of epigenetic enzymes, further elucidating the roles of these specific molecules. We will also review literature supporting transgenerational inheritance of epigenetic changes associated with a history of drug intake. We conclude by discussing important future directions for research investigating epigenetic mechanisms associated with drug addiction.

2. The link between drug action, intracellular signaling, and epigenetic changes

Both endogenous neurotransmitters and drugs interact with neuronal proteins, such as neurotransmitter receptors, proteins involved in synaptic homeostasis (e.g., neurotransmitter metabolic enzymes, transporters, etc.), and proteins involved in intracellular signaling pathways. These intracellular signaling pathways can propagate to the cell nucleus, leading to changes in gene expression [6]. Often, the first change observed in the cell nucleus following an environmental perturbation (e.g., drug use, stress, novelty, etc.) is the expression of immediate early genes (IEGs). Common IEGs encode transcription factors that increase expression of other target genes by binding to the genes' promoter region, which is a sequence in the DNA that signals the cell to initiate transcription [7, 8]. IEGs are rapidly induced and are often used as a marker of changes in neuronal signaling activity [9]. Both IEGs and target genes may undergo epigenetic modifications that regulate their expression. Thus, either natural signaling in response to environmental stimuli or drug-induced changes in signaling can invoke epigenetic mechanisms that alter gene expression. The dynamics of the epigenetic changes may be specific to the degree and phase of drug exposure, where particular epigenetic marks may only arise (or disappear) following acute or chronic drug administration, or during a period of withdrawal from drug use [10].

3. DNA epigenetic modification

A given gene is composed of a sequence of nucleotide base pairs in the DNA that are unique to that gene. For coding genes, the DNA sequence of base pairs serves as the blueprint for making a particular protein. Given that proteins are the machinery for cell structure and function, gene expression changes in a neuron can alter cell protein composition and, in turn, change the way that the neuron functions and communicates with other neurons.

There are four different nucleotide bases that compose the sequence portion of the DNA molecule, including the pyrimidines cytosine (C) and thymine (T), and the purines adenine (A) and guanine (G). Due to the structures of these nucleotides, the chemical bond responsible for base pairing can only form between C and G or A and T, respectively. Cs followed by Gs in the DNA sequence (i.e., CpGs) can be modified by a reaction in which DNA methyltransferases (DNMTs) add a methyl group (CH3) to the 5-position of the C to form 5mC. Intracellular signals may initiate newly synthesized *de novo* DNA methylation, which is mediated by DNMT subtypes DNMT3a and DNMT3b. Subtype DNMT1, on the other hand, maintains DNA methylation patterns across cell replication, such that the newly synthesized DNA has the exact methylation pattern that existed before DNA replication. In general, DNA methylation is correlated with a decrease in DNA accessibility and therefore is thought to be a mechanism of silencing gene expression. Methylated DNA can silence gene expression by interfering with the binding of transcriptional activators or by binding to proteins with a methyl-CpG-binding domain (MBD), such as methyl-CpG binding protein 2 (MECP2), that then form a complex with other proteins that together repress DNA accessibility [11].

Historically, DNA methylation was believed to be a permanent modification. However, demethylation of DNA can occur and also contributes to dynamic changes in gene expression. While passive demethylation in dividing cells may be due to malfunctioning of DNMT1, active demethylation occurs in both dividing and nondividing cells by enzymatic reactions. One reaction changes 5mC into a T, which is then recognized as a G/T mismatch. The mismatch activates a base excision repair (BER) pathway that utilizes thymine DNA glycosylase (TDG) and ultimately replaces T with a nonmethylated C [12]. Another reaction catalyzed by 10–11 translocation enzymes (TET) adds a hydroxyl (–OH) group to 5mC forming 5hmC. 5hmC itself has effects on gene expression and it can undergo further reactions that convert it back to a nonmethylated C [13]. Therefore, demethylation of DNA is generally correlated with an increase in DNA accessibility.

4. DNA methylation changes associated with drugs of abuse

Although DNA methylation is typically a stable epigenetic process, drugs of abuse have been shown to alter both DNA methylation and its associated enzymes. Much of this research has focused on DNA methylation in the nucleus accumbens (NAc), a brain region involved in reward and motivation learning [14]. A well-established marker of repeated exposure to drugs of abuse is an increase in the transcription factor ΔFosB protein in the NAc [15]. Acute or repeated cocaine administration decreases methylation at the *fosB* promoter in the NAc of rodents, which co-occurs with increases in *fosB* mRNA expression [16]. This may serve as a mechanism by which exposure to drugs of abuse produces stable increases in ΔFosB protein expression. Acute or chronic cocaine administration also increases *Dnmt3a* mRNA and MeCP2 protein expression in the NAc [16–18]. These increases are accompanied by decreases in psychostimulant reward as measured by conditioned place preference (CPP), a procedure in which an animal experiences a drug state while confined to one compartment of an apparatus and a neutral state while confined to an alternate compartment during conditioning, resulting in a shift in the animal's preference for the drug-paired compartment when given free access to both compartments. The decreased CPP effects are believed to be mediated by *Dnmt3a*- and MeCP2-induced silencing of genes that encode proteins that are needed for adaptation and functioning of NAc neurons [17, 18]. In addition, the TET enzyme that catalyzes DNA demethylation and subsequent transcriptional activation via conversion of 5mC to 5hmC [19] is decreased in the NAc in both rodents following cocaine administration and in postmortem tissue from human cocaine addicts [20]. Paradoxically, this decrease in TET is associated with increases in 5hmC expression at specific gene loci that have previously been linked to addiction [20]. Further investigation is needed to explain this complex pattern of epigenetic changes.

5. Histone modification

In order for the long strands of DNA to fit within a cell's nucleus, DNA is tightly condensed into chromatin. Chromatin is made up of nucleosomes that contain a histone protein core comprised of two copies of each of four different histone proteins, H2A, H2B, H3, and H4, as

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well as 147 base pairs of DNA that is wrapped around the histone core (**Figure 1**). Chromatin can either be tightly (i.e., heterochromatin) or loosely packaged (i.e., euchromatin), where the former restricts and the latter permits gene expression. Chromatin is able to undergo dynamic remodeling by chemical modification of amino acid residues of the histone core proteins. Similar to DNA methylation, histone proteins can undergo post-translational addition or removal of one of several chemical groups via enzymatic reactions. There are more than 100 different posttranslational modifications that may occur and these changes correlate with either the activation or the suppression of gene expression.

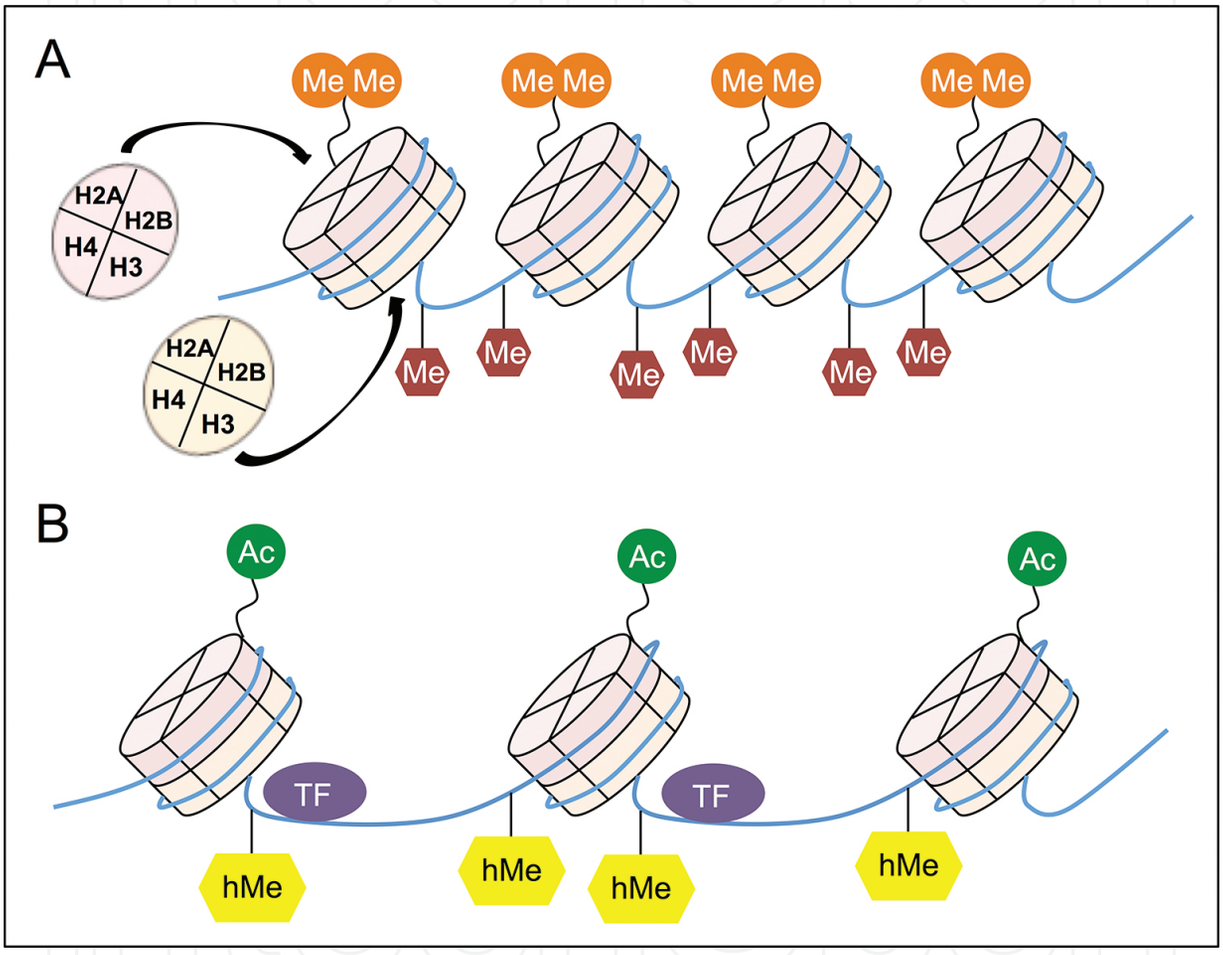

Figure 1. DNA and histone chemical modifications. DNA (blue lines) wraps around pairs of histone proteins H2A, H2B, 3, and 4 (light pink and tan) that form an octomer histone protein core. (A) Closed chromatin, due to DNA methylation (Me; red) and histone dimethylation on H3K9 (Me; orange), leads to transcriptional repression. (B) Open chromatin, due to DNA hydroxymethylation (hMe; yellow) and histone acetylation (Ac; green), allows for transcription factors (TF; purple) to recruit RNA polymerase for transcription initiation.

A powerful technique for studying post-translational histone modifications is chromatin immunoprecipitation (ChIP). ChIP utilizes antibodies that bind specifically to chemically modified histone proteins, which can then be isolated along with the associated DNA (i.e., promoter regions, gene bodies, etc.) from the rest of the tissue. Next, histones and DNA segments are denatured and levels of specific DNA sequences are measured. This technique

can be used to (1) determine which gene or gene promoter may be associated with a specific histone modification, (2) correlate changes in histone modification with expression of specific genes, and (3) suggest possible mechanisms for how a gene is turned on or off following an experimental manipulation (e.g., drug administration).

6. Histone modifications associated with drugs of abuse

6.1. Acetylation

Acetyl groups are added to histone proteins, typically on a lysine residue, by histone acetyl transferases (HATs). Addition of acetyl groups leads to a more relaxed, less condensed chromatin state by negating the positive charge of the histone protein that is attracted to the negatively charged DNA (**Figure 1B**). Indeed, increases in drug-induced gene expression often positively correlate with the levels of histone acetylation.

Previous work has found widespread changes in acetylation of histone H3 and H4 subunits in the NAc following acute and repeated psychostimulant administration in rodents [21, 22], suggesting that many genes in the NAc may be primed for transcription, while others are suppressed. Acute cocaine administration induces expression of the IEGs *c-fos* and *fosB* in rodents [16, 23], and ChIP analysis revealed increases in H4 acetylation at the respective promoter regions of these genes [21]. With repeated cocaine administration, the increase in *fosB* expression is maintained and is associated with increases in H3 acetylation at the *fosB* promoter region [21]. This mechanism likely contributes to ΔFosB protein accumulation in the NAc following repeated drug exposure. Repeated cocaine administration also reduces the ability of cocaine to induce *c-fos* and this is accompanied by a reduction in H4 acetylation at the *c-fos* promoter region [21, 24]. Furthermore, chronic opiate administration decreases expression of another IEG, brain-derived neurotrophic factor (*Bdnf*), and decreases H3 acetylation at the *Bdnf* promoter in the ventral tegmental area (VTA) [25]. *Bdnf* is critical for the development and maintenance of synaptic structure and function [26, 27] and drugs of abuse exert their reinforcing effect primarily by activating mesocorticolimbic dopamine neurons that originate in the VTA and project to the NAc, prefrontal cortex (PFC), amygdala, and hippocampus [28, 29].

Additionally, alcohol withdrawal in rodents reduces expression of the IEGs activity-regulated cytoskeleton protein (*Arc*) and *Bdnf* in the amygdala along with decreases in H3 acetylation at the respective gene promoters [30]. The amygdala is involved in processing emotional memories and it plays a critical role in alcohol-related behavior and anxiety [31]. Although these examples suggest functional links between degree of acetylation and associated gene expression, histone acetylation may occur even without changes in gene expression [32]. Therefore, further work into the causal role of acetylation in drug-induced gene expression is required.

6.2. Methylation

In contrast to histone acetylation, methylation can be associated with both transcriptional activation and repression, depending on which histone residue is modified. For example, dimethylation of histone H3 Lysine 9 (i.e., H3K9me2) is commonly associated with transcriptional repression. One histone methyltransferase that catalyzes H3K9me2, G9a, is decreased in the NAc following both chronic cocaine and opiate administration [33, 34]. It should be noted that this also occurs following chronic social stress in mice that produces depressive-like behavioral phenotypes, including decreased social interaction and increased anhedonia [35]. Similarly, G9a is decreased in postmortem NAc tissue of clinically depressed patients [35]. Decreases in G9a are associated with increases in cocaine and morphine CPP [33, 34]. Interestingly, G9a opposes expression of ΔFosB [33, 34]. In turn, ΔFosB represses G9a expression, creating a feedback loop that perpetuates its own expression through disinhibition. Similarly, postmortem NAc brain tissue of human cocaine addicts exhibits decreases in G9a expression [36] and increases in ΔFosB expression [37], providing further support for a functional link between these two molecules. In addition to specific genes, compelling work using ChIP and high-throughput sequencing of the associated genome has shown that cocaine-induced downregulation of H3K9me2 expression preferentially occurs in nongenic regions of chromosomes [38], suggesting additional roles of histone methylation that may be independent of traditional effects on specific protein-coding genes.

7. Noncoding RNAs

Before the 1990s, noncoding RNA was often referred to as "junk DNA" that was thought to have little relevance to biological function. A growing body of research over the past 25 years has shown that noncoding RNAs have pivotal roles in almost every cellular process investigated. One class of noncoding RNAs that has received much attention is microRNAs (miRNAs). miRNAs are small transcripts (~22 nucleotides) that regulate gene expression post-transcriptionally. They are transcribed from DNA in a manner similar to protein-coding transcripts, where transcription factors recognize promoter sequences upstream of miRNA genes and initiate transcription (**Figure 2**). Once transcribed, the several hundred nucleotide long transcript folds and binds to itself, producing a stem-loop hairpin structure referred to as a pri-miRNA. The enzyme, Drosha, trims the pri-miRNA into a smaller form known as pre-miRNA (~70 nucleotides long). Pre-miRNA is then transported out of the nucleus into the cytoplasm, where the loop portion of the pre-miRNA is cleaved by the enzyme Dicer [39]. Now as a double-stranded RNA molecule, it is unwound and one strand joins with several proteins to form the miRNA-induced silencing complex (miRISC). The mature miRNA binds to complementary sequences in the 3′ untranslated region (UTR) of a target mRNA where miRISC causes either translational repression, deadenylation, or endonucleolytic cleavage of the target mRNA, preventing its expression [40]. Importantly, the mature miRNA needs only ~6–8 complementary nucleotides for which to base pair with the 3′ UTR of the target mRNA, and therefore, one miRNA can target several hundreds of different mRNAs in a given cell. For this reason, miRNAs have been regarded as "master regulators" of gene expression. Changes

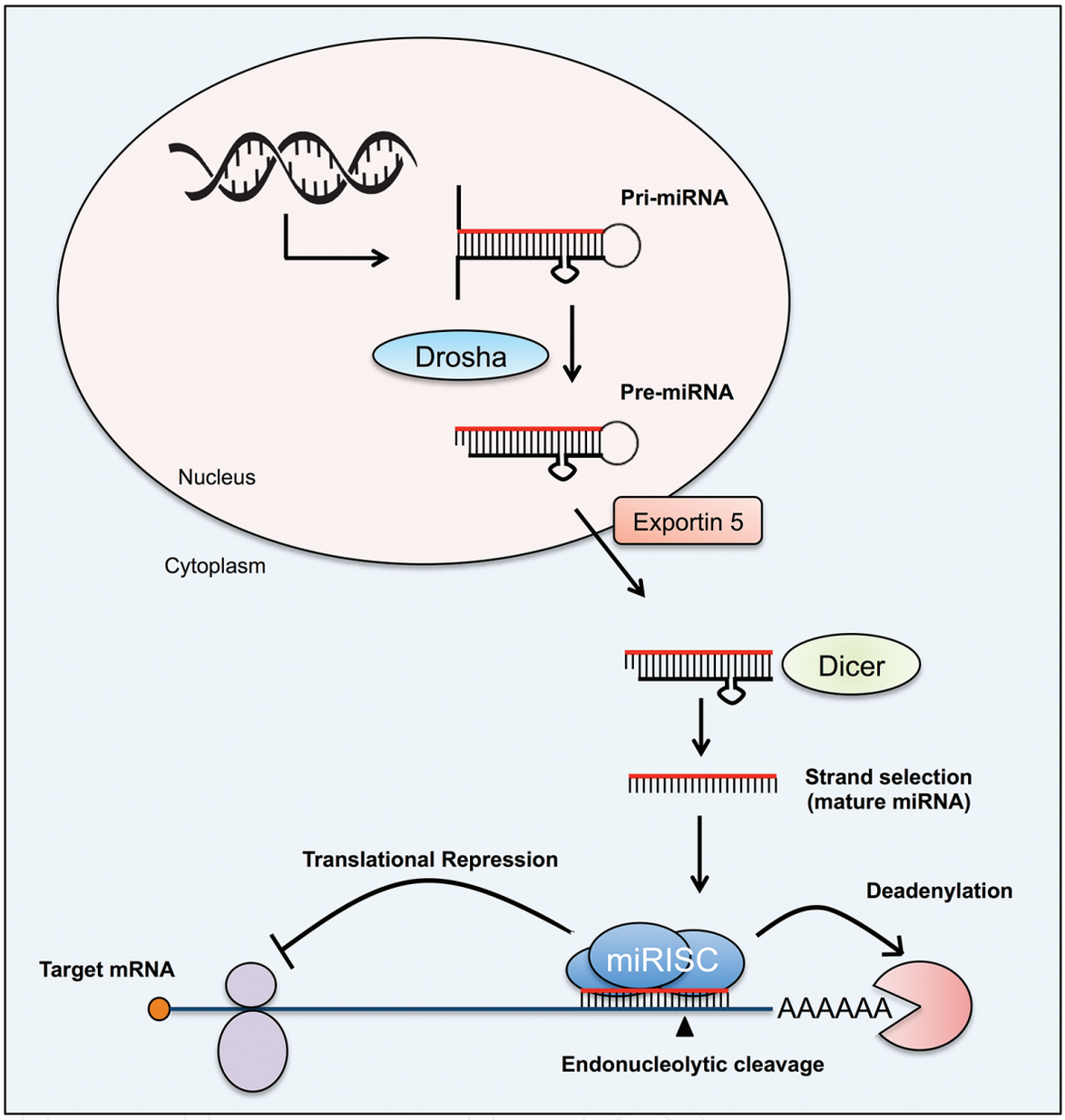

Figure 2. MicroRNA processing and function. MicroRNAs are transcribed similarly to protein-coding RNAs, except they form a stem-loop structure following transcription (i.e., pri-miRNA). The enzyme, Drosha, trims the ends of the stem (i.e., pre-miRNA) to prepare for exportation from the nucleus via Exportin 5. Once in the cytoplasm, Dicer cleaves the loop of the pre-miRNA that produces a double-stranded RNA. One strand (i.e., mature miRNA) is chosen to be incorporated into the miRNA-induced silencing complex (miRISC). Upon binding to a complementary sequence in the 3′ untranslated region of a target mRNA, either translational repression, deadenylation, or endonucleolytic cleavage may occur. All three mechanisms lead to decreases in protein product.

in miRNA expression can therefore lead to widespread changes in gene expression and alteration in several cellular signaling cascades. Other types of noncoding RNAs include: (1) PIWI-interacting RNA (piRNA), which regulates sperm development, (2) small nuclear RNA (snRNA), which regulates mRNA splicing, and (3) long noncoding RNA (lncRNA), which has widespread effects on chromatin modification and transcription [41].

8. Noncoding RNA changes associated with drugs of abuse

Given that most drug abuse research has focused on miRNAs, we will focus on this subclass. One approach to finding candidate addiction-related miRNAs is to examine miRNA expression changes within brain regions implicated in addiction following varying levels of drug exposure. Using this approach, Hollander and colleagues [42] found that rats given extended (6 h/day), but not restricted (2 h/day), access to cocaine self-administration exhibited upregulation of miR-212 in the dorsal striatum, a region involved in establishing habitual behavior [43]. Since the extended access self-administration model produces a behavioral phenotype that mimics the escalation of drug intake observed in human drug addicts, the findings suggest that upregulated miR-212 may play a role in the development of compulsive drug taking. One gene target of miR-212 is MeCP2 [44], a protein whose increased expression in the NAc is associated with reductions in amphetamine reward CPP [17]. However in the dorsal striatum, decreases in MeCP2 via miR-212 regulation are associated with decreases in compulsive-like cocaine self-administration [44]. These findings highlight the importance of examining the roles of epigenetic modulators across different drug classes, brain regions, and drug abuse models.

Another approach to identify candidate miRNAs is through bioinformatics. Databases exist that identify predicted targets of miRNAs and their distribution within the brain. We recently identified miR-495 as a lead candidate that has targets enriched in the Knowledgebase of Addiction-Related Genes database [45] and exhibits high expression in the NAc [46]. We found that cocaine self-administration decreases levels of NAc miR-495 and increases expression of several addiction-related genes. These effects suggest that cocaine dysregulates NAc miR-495, leading to disinhibition of addiction-related gene expression.

miRNAs and other noncoding RNAs have also been implicated in brain changes observed with other drugs of abuse. Alcohol-dependent rats exhibit increases in miR-206 in the medial PFC (mPFC) [47], a brain region involved in executive control of drug-seeking behavior [48]. miR-206 directly targets and suppresses BDNF expression in the mPFC [47], where increases in BDNF in this region are associated with inhibiting motivation for cocaine [49, 50]. This suggests increases in miR-206 likely contribute to the development of alcohol dependency through suppression of BDNF. Additionally, several lncRNAs exhibit expression changes in the NAc of heroin addicts postmortem [51]. These promising findings suggest that noncoding RNAs provide a treasure trove of novel targets for regulating addiction-related gene changes and behaviors.

9. Pharmacological manipulations of epigenetic mechanisms

Pharmacological agents that target specific epigenetic machinery have been used to further understand the role of epigenetic mechanisms in the effects of drugs of abuse and to explore their potential use as treatments for drug addiction. Most preclinical studies have utilized both

systemic and intracranial administration of these compounds, where the former has a more human translational value, while the latter allows for greater brain region specificity.

9.1. Methyl supplementation and DNMT inhibitors.

DNA methylation can be altered pharmacologically by using methionine or DNMT inhibitors. Methionine is an amino acid commonly found in diet, where methionine metabolism yields methyl groups that serve as donors for methylating DNA. DNMT inhibitors exert the opposite effect by preventing DNMT from catalyzing DNA methylation. Daily, systemic administration of methionine has been shown to reduce both the rewarding and motivating effects of cocaine in rodents [18, 52]. In contrast, intracranial administration of a DNMT inhibitor (i.e., RG108) into the NAc increases the rewarding effects of cocaine [18]. However, this same manipulation decreases drug-seeking behavior following a prolonged abstinence period [53]. These findings suggest that the effect of DNA methylation in the NAc may depend on whether or not there has been a period of abstinence following cocaine exposure. Indeed, our lab and others have shown that dynamic changes occur during forced abstinence from cocaine in animal models, and that these changes can result in opposing effects of pharmacological challenge on cocaine abuse-related behavior depending on whether the manipulation occurs during active drug intake versus abstinence [54–56]. It should be noted that work using DNA methyl supplementation and DNMT inhibitors has primarily been done with cocaine and needs to be tested on other drug classes.

9.2. Histone deacetylase inhibitors

The removal of an acetyl group from a histone is catalyzed by histone deacetylases (HDACs). This reaction results in condensing the chromatin and repressing transcription. HDAC inhibitors prevent this reaction from occurring, thereby maintaining DNA accessibility. There are five different classes of HDACs (e.g., I, IIa, IIb, III, and IV) and each class contains multiple HDAC enzymes (e.g., HDAC1, HDAC8, SIRT1, etc.). HDAC inhibitors range in their selectivity for specific HDAC classes. Drugs that target both class I and II HDACs (e.g., Tricostatin A, sodium butyrate, and SAHA) have been found to enhance cocaine locomotor sensitization [21, 57, 58], cocaine and opiate CPP [21, 58, 59], and cocaine self-administration [60] when administered systemically prior to cocaine exposure. In contrast, administration of HDAC compounds *following* cocaine exposure attenuates cocaine CPP [61]. Similarly, these compounds appear to produce mixed effects with alcohol, with some reporting increases [62] and others reporting decreases [63, 64] in consumption. While these effects were found during active drug administration, HDAC inhibitors have also been shown to alleviate anxiety symptoms during alcohol withdrawal [30, 65]. Additionally, several studies have found that the effects of the class I/II HDAC inhibitors were specific to drug self-administration, as no effects were found with these drugs on food reinforcement [60, 63, 64]. Collectively, it appears that class I/II HDAC inhibitors can produce both increases and decreases in drug-abuse-related behavior, and that the effects may vary depending on whether testing occurs during drug exposure or withdrawal.

More consistent effects have been observed with selective HDAC inhibitors. For instance, the selective class I HDAC inhibitor, MS-275, decreases both alcohol and cocaine abuse-related behavior in rodents [63, 64, 66, 67]. Also, the highly selective HDAC3 inhibitor, RGF-P966, decreases cocaine CPP [68]. These findings suggest that the use of more selective HDAC inhibitors may improve behavioral outcomes.

10. Genetic tools for uncovering epigenetic roles in drug-abuse-related behavior

While pharmacological approaches have translational value for development of therapeutic agents, efficacy may be compromised by the widespread drug distribution if the effects of an epigenetic manipulation vary depending on the brain region of interest. Also, pharmacological manipulations used to date have widespread effects on the genome, whereas sharpening the mechanism/location targeted may improve desired outcomes. Recent preclinical research has shed light on this area with technologies that selectively manipulate genes in specific brain pathways and cell types.

10.1. Viral vectors

One approach to manipulating a certain gene within a particular brain region is the use of viral vectors. Viral vectors are constructed to be nonreplicative so that they do not produce more viral particles after infecting the cell. They enter the cell through endocytosis and insert a gene of interest (i.e., transgene) into the genome of specific neurons (**Figure 3**). There are many different modes of transfection that vary in length from days to months. In order to achieve high levels of expression in a particular cell type, within the viral vector the transgene is typically downstream from a promoter sequence that is specific to that cell. Thus, upon viral transfection, the cells own transcriptional machinery will recognize and bind to the promoter that will then activate transcription of the transgene. The direction of regulation (i.e., increase vs. decrease expression) is determined by the sequence of the transgene. For instance, an increase in gene expression is obtained by inserting the sequence of the transgene into the viral vector with a strong upstream promoter. In order to decrease gene expression, a couple of methods may be used. One involves transfecting a short-hairpin RNA (shRNA) that is processed into a mature short-interfering RNA (siRNA). siRNAs are similar to miRNAs, except that they are perfectly complementary to the target mRNA and will therefore selectively downregulate only one target gene, in contrast to the multiple targets of most miRNAs. This is referred to as a 'knockdown,' rather than a 'knockout,' as it is preventing translation of the gene rather than completing deleting it from the genome. In order to accomplish a 'knockout' using viral vectors, transgenes that express a new gene editing approach, called the CRISPR-Cas9 system, can be used. The latter uses a guide RNA that is complementary to specific sequences in the DNA (e.g., gene of interest) that directs enzymes to that site and excises the sequence from the DNA, therefore deleting it.

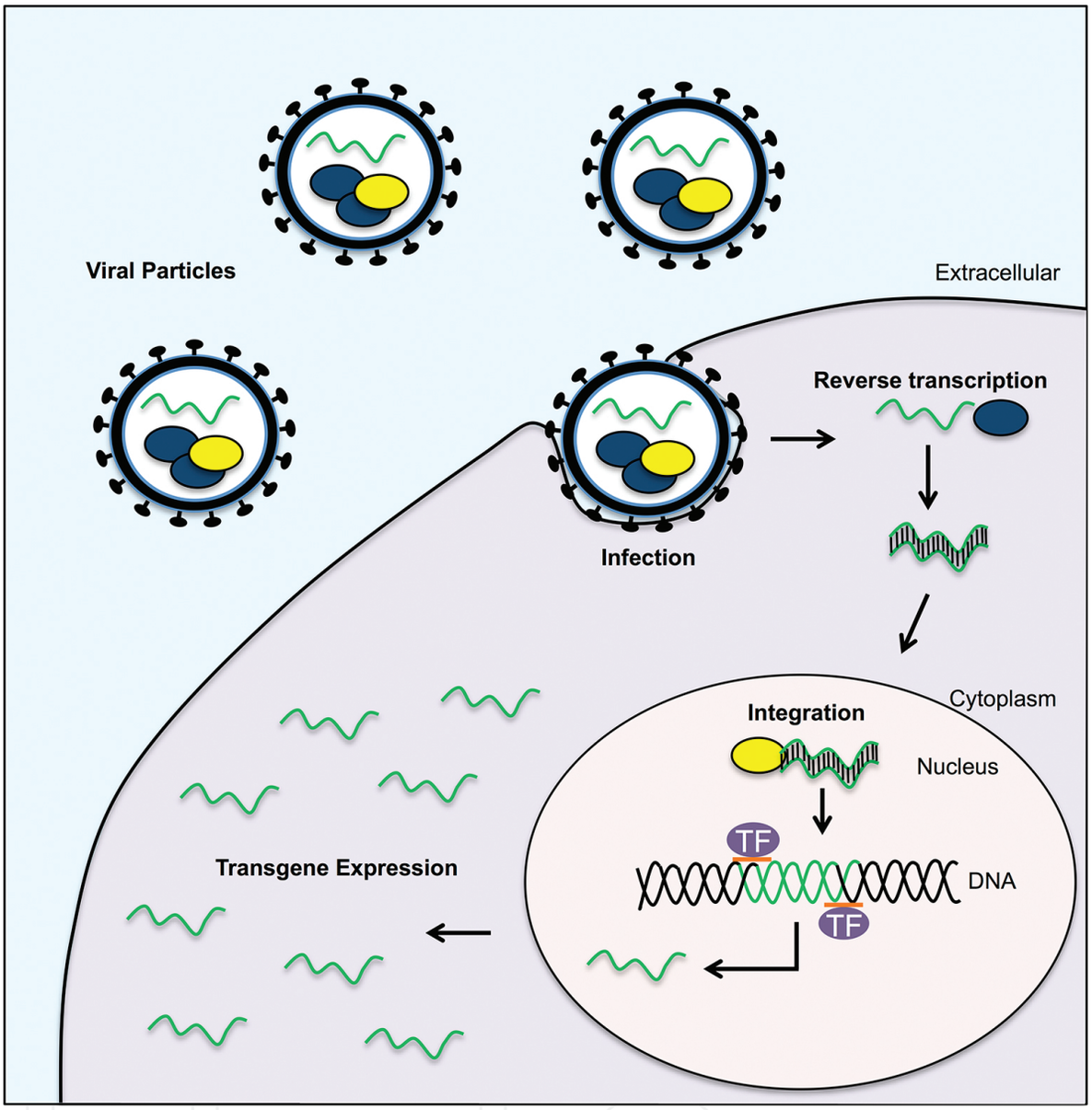

Figure 3. Viral-mediated gene transfer. Viral particles are infused into a region of interest and infect local cells through receptor-mediated endocytosis. Once viral particles are released from the vesicle inside the infected cell, viral RNA is reverse-transcribed into DNA (via reverse transcriptase; dark blue) and transported into the nucleus, where it becomes integrated into the genome (via integrase; yellow). By using a strong promoter (orange line) upstream of the transgene, the cell's transcriptional machinery produces an abundance of viral transgene expression in the cell. TF = transcription factor.

Research using viral vectors has furthered our understanding of the impact of epigenetic manipulations on drug-abuse-related behavior. As previously described, DNA methylation is thought to inhibit cocaine abuse-related behaviors in animal models [16, 18, 52]. To test whether *Dnmt3a* expression in the NAc specifically mediates these effects, LaPlant et al. [18] infused viral vectors into this region that either increased or decreased *Dnmt3a* levels. Increasing NAc *Dnmt3a* expression countered cocaine CPP in mice, while decreasing expression increased this behavior [18]. Interestingly, this same manipulation also increases depressive-

like behavior following repeated social stress in mice [18], suggesting the blunted rewarding effects of cocaine may be due to increases in anhedonia. This illustrates the importance of testing the role of epigenetic modulators in both drug abuse and mood disorder models.

Another exciting use of viral vectors is to express synthetically engineered transcription factors that bind to specific sequences in the DNA and regulate histone modifications at one specific gene loci. Heller and colleagues [69] recently used this approach and found that histone acetylation or methylation near the *fosB* gene locus increases or decreases cocaine reward CPP, respectively. Again, this same manipulation produces either anti- or pro-depressive behaviors, respectively, following repeated social stress [69], further demonstrating the complex role of these molecules in both reward and emotional regulation processes. Bidirectional manipulation of cocaine self-administration in rats has also been demonstrated for miR-212 levels in the dorsal striatum where viral-mediated increases prevent escalation of cocaine self-administration, whereas knockdown increases cocaine self-administration [42]. In some cases, decreasing miRNA levels may be needed to attenuate addiction-related behavior. For instance, viral-mediated increases in miR-206 expression in the prefrontal cortex create an alcohol-dependent phenotype in rats [47], and, therefore, it is possible that decreasing miR-206 levels in the PFC may be protective against alcoholism. These examples suggest that the development of new therapeutics that target epigenetic mechanisms have potential for treating addiction. Currently, there are no pharmacological agents for manipulating miRNAs, although development is in the initial stages for their delivery in drug compounds [70]. A future challenge for this avenue of research will be to develop methods of site-selective drug delivery.

10.2. Cre-Lox recombination

Another approach to manipulating gene expression is the use of Cre-Lox recombination (**Figure 4**). Cre recombinase is an enzyme that identifies sequences in the DNA called LoxP sites. When Cre recognizes these sites, it catalyzes a reaction that can either excise or invert the DNA sequence contained between the two sites, depending on which direction the LoxP sites are oriented. If the two LoxP sites are in the same direction, Cre will excise the DNA, effectively deleting a gene that is between those two sites. If the LoxP sites are in the opposite direction, Cre will then invert the two LoxP sites along with inverting the flanked DNA sequence. This latter effect allows for gene activation, where a previous nonfunctional inverted gene sequence becomes functional after Cre-Lox mediated-inversion.

Cre-lox recombination is carried out in rodents that are bred to have LoxP sites at specific locations in the DNA that flank a gene of interest (e.g., $Bdnf^{fl/fl}$). A viral vector expressing Cre recombinase can then be infused into a specific brain region and Cre-expressing infected cells will recognize the LoxP sites and either excise or invert the flanked gene. This will result in region- and temporal-specific manipulation of gene expression. Another approach with even greater precision involves breeding mice to express Cre in only certain cell types. This is accomplished by breeding rodents that express Cre downstream from a promoter that is specific for only one type or subtype of cells. For instance, Cre can be expressed specifically in catecholamine neurons when used downstream of a promoter for tyrosine hydroxylase (TH). TH is an enzyme involved in synthesizing catecholamines (e.g., dopamine). Only cells with

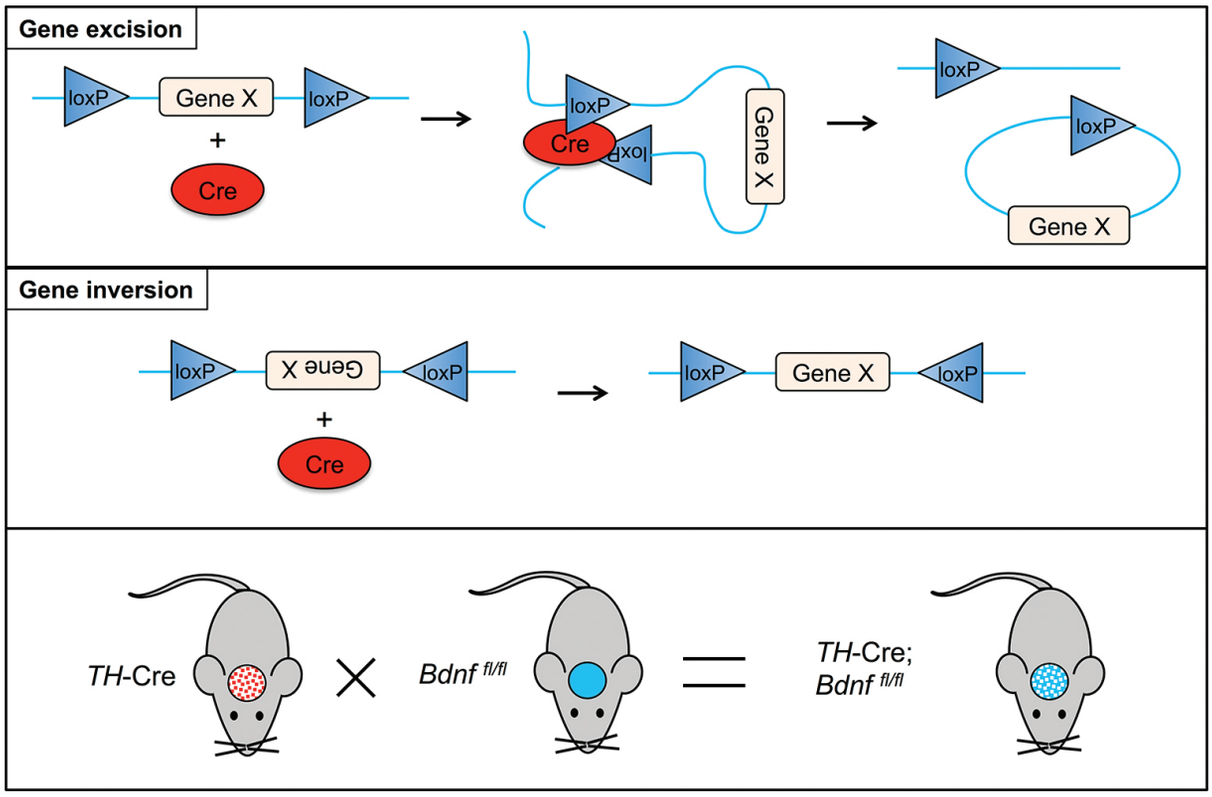

Figure 4. Cre-lox recombination. The top panel illustrates how Cre-lox recombination can result in gene excision, where Cre recombinase (red) recognizes two loxP sites in the same orientation and joins the two ends of DNA, then cleaves one end to join the other, resulting in excision of the flanked gene between the two loxP sites. The middle panel illustrates how Cre recognizes two loxP sites in opposite orientations and inverts the intervening DNA sequence (e.g., Gene X). Gene inversion can be used to turn on a gene that is initially inverted and inactive. The bottom panel depicts how crossbreeding two transgenic mice that express Cre recombinase only in tyrosine hydroxylase-expressing cells (*TH*-Cre) with mice that ubiquitously express Bdnf with flanked loxP sites ($Bdnf^{fl/fl}$) results in mice with deficient *Bdnf* expression only in *TH*+ cells.

TH will have the transcriptional machinery to recognize the TH promoter and express Cre. Next, there are two methods for manipulating gene expression in a cell-type-specific manner. The first is to crossbreed two transgenic mice: the one that expresses Cre only in certain cell types (e.g., TH+ neurons) and the other that ubiquitously expresses a LoxP-flanked *Bdnf* gene (i.e., $Bdnf^{fl/fl}$). The offspring will no longer express *Bdnf* in TH-expressing cells. A limitation of this technique is that Cre recombination occurs at conception and the transgene is either expressed or deleted permanently. Therefore, changes may occur during development to compensate for the gene modification, making it difficult to know whether subsequent functional differences are due to the gene modification or the compensatory changes that ensued thereafter. Another way to overcome this limitation is to inject a viral vector into a brain region that contains the gene of interest in a plasmid with the gene flanked by inverted LoxP sites. While the virus will infect all the cells in that region, only the cells that are expressing Cre recombinase (e.g., TH+) will recognize the LoxP sites. In this case, Cre recombination will only occur in specific cell types in a particular brain region and, importantly, during a specific time point during development.

Research employing the Cre-Lox recombination approach has shown that the effect of epigenetic mechanisms can be cell-type specific. For instance, the histone methytransferase *G9a* has differential roles in cocaine-related behaviors depending on whether it is expressed in striatal neurons that contain dopamine D1 (D1R) versus D2 (D2R) receptors. *G9a* is down-regulated by cocaine in both D1R and D2R-containing neurons; however, Cre-mediated downregulation of *G9a* selectively in D1R-neurons is associated with decreasing cocaine CPP and locomotor behavior in mice, while the opposite effects occur with selective down-regulation in D2R-neurons [36]. These effects were observed using both Cre-Lox recombinase procedures described above, providing strong evidence for the cell-type-specific role of *G9a* in cocaine abuse-related behavior.

11. Transgenerational epigenetic inheritance of addiction-like phenotypes

Perhaps the most intriguing discovery in epigenetics is that epigenetic marks acquired due to experience can be passed along to future generations. Unfortunately, this may include epigenetic changes that make one vulnerable to addiction. The phrase "it runs in the family" is often spoken in social circles regarding the seeming ability of addiction to be inherited. While much is known about inheritance based on classical Mendelian genetic inheritance, much less is known about transgenerational epigenetic inheritance.

Several criteria must be met for transgenerational epigenetic inheritance. First, in order to pass down epigenetic changes across generations, the changes need to be present in the germ cells (i.e., sperm or egg). In other words, the epigenetic changes must occur in future generations independent of behavioral and social transfer, relying only on the molecular transmission of epigenetic information [71]. Second, the behavioral phenotypes need to persist across several generations, depending on the sex and pregnancy status of the parent exposed to the initial environmental trigger. In males and nonpregnant females, an environmental trigger that affects the parent generation (i.e., F0) and their germ cells, will directly impact the next (i.e., F1) generation. This is referred to as multi- or inter-generational inheritance [72]. However, if the behavioral phenotype persists into the third generation (i.e., F2), which had no direct exposure to the trigger, it can be regarded as transgenerational inheritance. With pregnant females, not only is the parent and embryo directly affected, but also the germ cells of the embryo that will develop into the F2 generation. Therefore, the F3 generation must exhibit the phenotype to be considered transgenerational. Third, epigenetic modifications present in the parents need to persist into future generations (see **Figure 5**). Interestingly, most epigenetic marks (particularly DNA methylation) are erased immediately in the embryo following fertilization [73]. Very few exceptions are currently known, but some include imprinted genes (i.e., methylation-induced silencing of genes in one parent's allele and not in the others), certain histone and protamine (i.e., histone-like proteins found in sperm) modifications, and reserve pools of coding and noncoding RNA [72]. Although narrowing the field of investigation, the complex pattern of changes required for transgenerational epigenetic inheritance still remains poorly understood [72, 74].

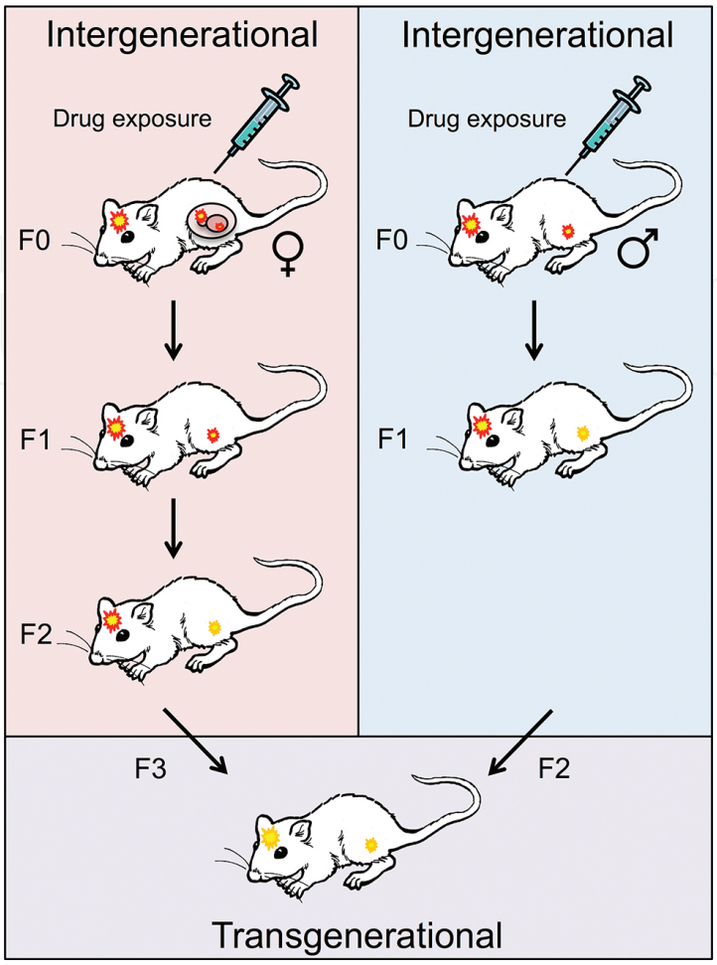

Figure 5. Epigenetic inheritance. For pregnant F0 females (top left panel), drug exposure directly affects (red-outlined symbols) both the F0 female and the fetus, including the brain and germ cells of the upcoming F1 generation. However, the F2 generation also receives direct effects of drug exposure from the F0 generation via the germ cells of the F1 generation. For males (top right panel), drug exposure directly affects both the F0 generation and the germ cells that lead to the F1 generation. Therefore, the F3 and F2 generation of the pregnant female and male, respectively, can receive transgenerational epigenetic inheritance from the F0 generation without having been in direct contact with drugs of abuse (orange-outlined symbols).

11.1. Drug abuse-related traits passed across generations

The idea that addiction-like phenotypes can be passed down across generations based on experiences of the parents is compelling in terms of uncovering potential biomarkers that could be used to predict one's risk of developing drug addiction. Only a few studies have investigated this possibility in the context of drug abuse models. Vassoler and colleagues [75] found that male adult rats with a history of cocaine self-administration passed an addiction-*resistant* phenotype onto male, but not female, offspring. One potential mediator of these effects is mPFC BDNF expression, where both the male F0 generation's sperm and the mPFC of the male, but not female, offspring exhibited increased H3 acetylation at the BDNF promoter, as well as increased BDNF expression in the mPFC of the male offspring. Consistent with this idea, mPFC BDNF is associated with resilience to drug effects [47, 49, 50]. These data are

particularly compelling given that it was the father who received the initial trigger (i.e., drug exposure), thereby avoiding potential confounds of maternal care, social/behavioral transfer, and *in utero* environment changes that may occur following drug exposure in the females. Nonetheless, one cannot rule out potential stress effects during copulation. Gapp et al. [76] avoided this potential confound by isolating the sperm of the affected F0 males and artificially inseminating the F0 females. They found that sperm noncoding RNAs from fathers subjected to early life stress sufficiently passed on molecular and behavioral phenotypes to the next two generations. Interestingly, early life stress is also a strong predictor for developing drug addiction [1]. Other studies have shown that repeated morphine administration in F0 female adolescents produces male offspring that are more sensitive to the analgesic and tolerance effects of morphine [77]. Furthermore, ethanol exposure to F0 males decreases ethanol intake and increases sensitivity to the inhibitory effect of ethanol on anxiety-like behavior in F1 offspring [78]. Reduced methylation at the *Bdnf* promoter was also observed in this study in both the F0 male sperm and in the F1 males' VTA. The VTA sends dopaminergic projections to the NAc and BDNF in the VTA has a facilitating effect on drug-abuse-related behavior [79]. These few examples provide some evidence of multi-generational inheritance of drug abuse-related traits and associated changes to the epigenome. However, further research is required to examine if these traits persist into additional generations and whether blocking or reversing the epigenetic changes in the germline will prevent transmission of these traits. The latter effect would have very exciting implications for approaches to prevent the development of addiction in future generations.

12. Concluding remarks

The studies reviewed provide compelling evidence for a link between drug-induced epigenetic regulation of gene expression and drug abuse-related behavior in animals. The epigenetic changes in gene expression occur in brain regions involved in reward learning and motivation. This leads to plasticity-related changes within the neurocircuitries that mediate these processes and is associated with aberrant behaviors that resemble hallmark symptoms of human drug addiction, such as escalation of drug intake [42, 44, 80, 81] and increased willingness to exert effort to obtain drug [46, 82]. Given that epigenetic mechanisms can produce long-lasting changes in gene expression, they are likely candidate explanations for the persistent nature of drug addiction in humans.

Current understanding of experience-dependent epigenetic changes is in its infancy. One notable limitation in this field is the dearth of research on drugs of abuse other than psychostimulants and alcohol. This is a particularly important gap to fill given that regulatory changes in addiction-related genes can have opposite effects on abuse-related behaviors depending on drug class [25, 83, 84]. The field also faces many challenges in discerning the involvement of epigenetic mechanisms in drug addiction given the vast number of molecular regulatory events that are altered by stress and drug experience, the complex interactions that can occur among these regulatory events, and their drug-, region-, and time-specificity. Indeed, high comorbidity exists between drug addiction and stress-related emotional disorders (e.g., PTSD

and depression) [85, 86]; therefore, additional work is needed to test whether epigenetic factors underlie the co-occurrence or are specific for one disorder over the other. It will be vital to test the generalizability versus specificity of epigenetic modifications in drug abuse-related behavior.

Despite the challenges that lie ahead, growing knowledge in this field will provide opportunities for novel preventions and interventions of drug abuse and dependence. New technologies for identifying and specifically targeting epigenetic processes hold promise not only for understanding the complex interactions between drug exposure, life experiences, and gene expression, but also as treatment strategies designed to counter epigenetic dysregulation. For instance, the use of synthetic transcription factors may allow drug-induced or inherited epigenetic marks to be erased in order to increase resilience when one is exposed to addictive drugs [69]. Future epigenetics research may also identify biomarkers of vulnerability that may aid prevention strategies. Collectively, these new avenues of drug abuse research are exciting given the urgent need for better treatments of this devastating disorder.

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