Epigenetic Effects of Cannabis Exposure

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ABSTRACT
The past decade has witnessed a number of societal and political changes that have raised critical questions about the long-term impact of marijuana (Cannabis sativa) that are especially important given the prevalence of its abuse and that potential long-term effects still largely lack scientific data. Disturbances of the epigenome have generally been hypothesized as the molecular machinery underlying the persistent, often tissue-specific transcriptional and behavioral effects of cannabinoids that have been observed within one’s lifetime and even into the subsequent generation. Here, we provide an overview of the current published scientific literature that has examined epigenetic effects of cannabinoids. Though mechanistic insights about the epigenome remain sparse, accumulating data in humans and animal models have begun to reveal aberrant epigenetic modifications in brain and the periphery linked to cannabis exposure. Expansion of such knowledge and causal molecular relationships could help provide novel targets for future therapeutic interventions.

Keywords: Addiction, Cannabinoids, CB₁ receptor, DNA methylation, Epigenetics, Neurodevelopment
http://dx.doi.org/10.1016/j.biopsych.2015.09.014

Extensive political and societal debates are currently being waged at state and federal levels regarding the legalization of marijuana (Cannabis sativa), which remains today the most commonly used illicit substance in the United States and in many countries worldwide. As evident in Figure 1, there has been a dramatic exponential increase of cannabis studies over the past two decades in response to the transformative implications resulting from the growing discussions and laws passed regarding legalization of recreational and medical marijuana use. Of the published studies to date in the PubMed database, about 13% relate to the neurobiological effects of cannabis and approximately 27% are directed toward obtaining behavioral insights. Despite the perceived low health risk of cannabis use by the general public, there is growing clinical awareness about the spectrum of behavioral and neurobiological disturbances associated with cannabis exposure, such as anxiety, depression, psychosis, cognitive deficits, social impairments, and addiction (1–7). The acute intoxication induced by cannabis consumption is strongly linked with concerns about its direct effects on cognition and motor function, but a central issue relates to its long-term impact, especially when exposure occurs during critical periods of brain development. Key gaps of scientific knowledge pertain to the biological mechanisms that maintain persistent phenotypic and molecular alterations long after its acute use.

The major psychoactive cannabinoid within cannabis, Δ⁹-tetrahydrocannabinol (THC), targets the endocannabinoid (eCB) system, which plays a key role in the development of the brain and several other organs. In recent years, various human and experimental animal studies have evaluated the long-term impact of cannabis and cannabinoids on neurodevelopment, behavior, and several biological systems such as immunological mechanisms and reproductive processes [reviewed in (7–10)]. Moreover, behavioral abnormalities and molecular impairments in the brain have also been demonstrated to extend even into subsequent generations of offspring whose parents were exposed to cannabinoids before mating (11–15).

The epigenome provides a cellular fingerprint of environmental experiences, including drug exposure history, and thus is a highly relevant biological candidate expected to maintain persistent abnormalities and aberrant neuronal processing over time. The role of epigenetics in psychiatric disorders has been a major scientific focus during the past few years. According to the classic definition, “an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” [as proposed by Conrad Waddington in the 1950s (16,17)]—this view implies heritability resulting in a phenotype. In the molecular biological era of recent years, “epigenetic” typically has been used to refer to mechanisms that modulate gene expression without altering the genetic code. Our article provides an overview of research endeavors relevant to cannabis-related epigenetic mechanisms that could shed light about the biological processes that establish the molecular platform that maintains marijuana’s protracted effects on gene expression and ultimately behavior.

EPIGENETIC MECHANISMS
In a biological mechanistic context, knowledge of how gene expression is regulated by the cellular network of cis-acting elements and trans-acting factors has evolved substantially during the past decade. Generally, the interaction between
The gene body is less understood and may act as either a regulator of gene silencing, whereas methylation within transcriptional regulatory sequences has frequently been shown to influence gene expression, often associated with the binding of methyl-CpG binding domain-containing proteins (e.g., methyl-CpG-binding protein 2 [MeCP2]). The oxidation of 5mC to 5-hydroxymethylcytosine by ten-eleven translocation proteins can prevent access to DNA methyltransferases and thereby can maintain an unmethylated state of the promoter, leading to transcriptional activation (25). Interestingly, DNA methylation marks at specific gene loci have been shown to persist even during the maturation of germ cells (26,27) and thus are interesting candidates for the propagation of the long-term effects of cannabis throughout multiple generations.

**DNA Methylation**

The role of DNA methylation (Figure 2B) in the regulation of gene expression is still controversial and highly dependent on genomic location, developmental stage, cell type, or disease state. Historically, CpG methylation in promoter regions and transcriptional regulatory sequences has frequently been associated with gene silencing, whereas methylation within the gene body is less understood and may act as either a positive or a negative effector (21,22). Accumulating evidence now also indicates that DNA methylation in the brain is reversible and its distribution changes throughout neuronal maturation and aging in neurodevelopmental disorders, including addiction to drugs such as cocaine (23,24). Mechanistically, DNA methylation (5-methylcytosine [5mC]) is generated by DNA methyltransferases. At promoter regions, 5mC is often associated with the binding of methyl-CpG binding domain-containing proteins (e.g., methyl-CpG-binding protein 2 [MeCP2]). The oxidation of 5mC to 5-hydroxymethylcytosine by ten-eleven translocation proteins can prevent access to DNA methyltransferases and thereby can maintain an epigenetically modified state of associated genes. (A) Gene expression is regulated by a network of DNA elements (e.g., promoters) and trans-acting factors (proteins) that bind to the DNA that interact physically and functionally to generate appropriate messenger RNA (mRNA) transcript levels from a gene. The resulting balance can be disrupted by drug exposure. Regulatory mechanisms include DNA methylation (Me), positioning and posttranslational modifications of nucleosomes (small blue balls), recruitment of sequence-specific and basal transcription factors and RNA polymerase II, and noncoding RNAs. The DNA-protein structure forms three-dimensional structures (represented by the chromatin loop) that influence the expression of individual genes. (B) DNA methyltransferases (DNMT) generate 5-methylcytosine (pink stars) at CpG sites, facilitated by methyl-CpG binding domain (MBD)-containing proteins. Ten-eleven translocation (TET) proteins mediate the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (green stars), leading to demethylation of the DNA. (C) Modifications of nucleosomal histone tails such as methylation (Me) and acetylation (Ac) are mediated by histone methyltransferases (HMT) and histone acetyltransferases (HAT), respectively. Depending on modified amino acid residue, methylation can have either permissive (e.g., on lysine 4 [K4]) or repressive (e.g., on lysine 9 [K9]) effects on transcription. Permissive modifications facilitate gene activation via the recruitment of the RNA polymerase II machinery. Acetylation is removed by histone deacetylases (HDAC) and can lead to transcriptional repression. (D) MicroRNAs are produced from specific genes and target protein-coding mRNA for degradation, thereby preventing protein production.
Histone Modifications

On the protein level, the main epigenetic mechanism that has been implicated in neurobiological disturbances related to drug abuse is posttranslational modifications of nucleosomal histones (Figure 2C), which with the ~146 base pair of DNA that encircle them comprise the basic unit of chromatin. Histones are subject to a variety of modifications, including, but not limited to, lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation (28). These modifications occur primarily within the histone amino-terminal tails protruding from the surface of the nucleosome, as well as on the globular core region, and have been shown to influence both the accessibility of genomic regions and the binding of trans-acting factors to the DNA (29). Changes in acetylation and phosphorylation in response to drug exposure are often transient and appear to be associated with the quick activation of genes rather than the maintenance of an altered transcription state (30). However, histone lysine methylation is known to maintain stable gene expression alterations, and it is also the nucleosomal modification that has been associated with the long-term effects of marijuana and different cannabinoids in neurons and other cell types.

Noncoding RNAs

These functional RNA molecules are transcribed from DNA but are not translated into proteins. Many noncoding RNAs (ncRNAs) regulate gene expression at the transcriptional and posttranscriptional level. Those ncRNAs that are known to be involved in epigenetic processes can be divided into two main groups—short ncRNAs (<30 nucleotides) and long ncRNAs (>200 nucleotides). The three major classes of short ncRNAs are microRNAs (miRNAs), short interfering RNAs, and piwi-interacting RNAs (35). Of these, alterations in miRNA profiles have been associated with cannabinoid exposure in the mammalian brain, peripheral blood cells, and the gut (Figure 2D) (36–39). While the exact genomic targets of specific cannabinoid-affected miRNAs remain to be characterized, these observations are mechanistically intriguing given the variety of tissue-specific cellular and developmental processes that are influenced by miRNAs.

THE ENDOCANNABINOID SYSTEM

Cannabis targets the eCB system, which contributes to organogenesis as well as neurogenesis and gliogenesis of the central nervous system. It is well documented that the eCB system controls neuronal hardwiring during prenatal ontogeny, relevant to the development of neural pathways such as the cortico-striato-thalamic circuit, which are implicated in addiction and psychiatric disorders (40,41). During postnatal development, the eCB system is known to be a critical regulator of synaptic plasticity. In mammals, two cannabinoid receptors have been identified (type 1 cannabinoid receptor [CB1R] and type 2 cannabinoid receptor [CB2R]), along with two major endocannabinoids as their ligands, N-arachidonoyl-ethanolamine (anandamide) and 2-arachidonoyleglycerol (42). During development, these endogenous cannabinoid transmitters act as signaling molecules via a primarily autocrine activation of CB1Rs colocalized in the same developing neurons, whereas in the mature brain, eCBs are synthetized by postsynaptic neurons and travel retrogradely across the synapse to inhibit presynaptic neurotransmitter release via cannabinoid receptors (CBRs) (43). CB1R is the most abundant G-protein-coupled receptor in the adult brain and mediates in large part the neurobehavioral effects of THC (Figure 3). Consistent with the known neurobiological and behavioral effects of the eCB system, CB1Rs are abundant in brain areas involved in learning and memory (e.g., hippocampus), motor function (e.g., basal ganglia, cerebellum), and cognitive and emotional processes (e.g., striatum, amygdala, prefrontal cortex) (3), as well as the regulation of physiological and metabolic processes including feeding and stress response via the interaction of the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes (44,45). In neurons, CB1Rs are preferentially localized on the surface of presynaptic cells regulating both excitatory (glutamate) and inhibitory (gamma-aminobutyric acid) transmission. Low expression of CB2Rs has recently been reported in the brain, frequently in association with inflammatory processes (46), and it has been detected in neurons within mesocorticolimbic brain regions relevant to cognition and motor function (47,48). Despite its low abundance in the brain, modulation of the central nervous system is influenced by cannabinoid exposure.
system CB$_2$R has been implicated in addiction-related behaviors [49,50]. Both CBRs are present in peripheral tissues, including the immune system, adipose tissue, liver, skeletal muscle, and reproductive organs [51].

The normal epigenetic control of the eCB system has recently been reviewed [52]. In the current article, we focus on how cannabis, THC, and other exogenous cannabinoid receptor modulators alter epigenetic mechanisms and developmental regulation (Table 1). Briefly, however, various lines of evidence strongly suggest that the eCB anandamide and eCB signaling cascades mediated via CBRs regulate cellular functions in different tissues via epigenetic alterations in DNA methylation (e.g., cell differentiation in human keratinocytes, cells in the epidermis) [53], miRNA (regulating cells involved in interleukin production and inflammatory response) [38], and histone methylation (differentiation and inhibition of gliomagenesis) [31]. These data highlight the role of the eCB system in regulating a repertoire of cellular functions in diverse tissues through multiple epigenetic modifications and suggest that exogenous modulation of these pathways with drugs may have long-lasting neurobiological impact.

### EPIGENETIC MECHANISMS RELEVANT TO THE LONG-TERM EFFECTS OF CANNABIS

The study of epigenetics in relation to drugs of abuse has been a rapidly emerging field during the past several years, yielding important mechanistic revelations about different addictions and related neuropsychiatric disorders [54,55]. However,

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**Table 1. Epigenetic Alterations Related to the Effects of Cannabinoids in Different Organisms and Biological Systems**

<table>
<thead>
<tr>
<th>Cannabinoid</th>
<th>Epigenetic Alteration</th>
<th>Biological Target</th>
<th>Associated Effect or Consequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cannabis</td>
<td>Increased CpG DNA methylation at promoter</td>
<td>Human peripheral blood cells</td>
<td>Negative correlation between CB$_2$R methylation and mRNA levels in schizophrenic cannabis users</td>
<td>(59)</td>
</tr>
<tr>
<td>Cannabis</td>
<td><strong>H3K4me3, H3K9me2; Promoter, gene body</strong></td>
<td>Human adolescent peripheral blood cells</td>
<td>Less likely cannabis dependence and decreased risk of psychosis</td>
<td>(63)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>H3K4me3; H3K9me2; Promoter, gene body</td>
<td>Adult rat brain (NAC)</td>
<td>Decreased Drd2 gene mRNA levels in response to in utero THC exposure</td>
<td>(32)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>H3K9me3; H3K9me3; Promoter, gene body</td>
<td>Adult rat brain (NAC shell)</td>
<td>Increased Penk gene mRNA levels in response to adolescent THC exposure</td>
<td>(33)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>CpG DNA methylation at promoters, intergenic regions, especially in gene bodies</td>
<td>Adult rat NAC with parental THC exposure</td>
<td>Altered methylation enriched in genes implicated in synaptic plasticity</td>
<td>(15)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>H3K4me3, H3K9me3, H3K27me3, H3K36me3; Promoters, intergenic regions, especially in gene bodies</td>
<td>Differentiating mouse lymph node cells</td>
<td>Genome-wide alterations in histone modifications associated with dysregulated genes and noncoding RNAs</td>
<td>(34)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>Increased HDAC3 expression</td>
<td>Human trophoblast cell line BeWo</td>
<td>Gene dysregulation during placental development</td>
<td>(72)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>DNA methylation at CpG islands; miRNAs</td>
<td>Cerebellum and peripheral T cells of simian immunodeficiency virus-infected macaques</td>
<td>Altered DNA methylation, miRNA and miRNA expression profiles</td>
<td>(39)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>miRNAs</td>
<td>Mouse myeloid-derived suppressor cells</td>
<td>Altered mRNA, miRNA, and differentiation profile</td>
<td>(37)</td>
</tr>
<tr>
<td>THC$^{+}$</td>
<td>miRNAs</td>
<td>Intestine of simian immunodeficiency virus-infected macaques</td>
<td>Altered miRNA profile and intestinal epithelial cell composition</td>
<td>(36)</td>
</tr>
<tr>
<td>Exogenous Anandamide</td>
<td>Increased global DNA methylation</td>
<td>Spontaneously immortalized human keratinocytes (HaCaT cell line)</td>
<td>Decreased expression of differentiation-related genes and altered cell differentiation</td>
<td>(38)</td>
</tr>
<tr>
<td>Exogenous Anandamide</td>
<td>miRNAs</td>
<td>Mouse lymph node cells</td>
<td>Altered interleukin production and inflammatory response</td>
<td>(38)</td>
</tr>
<tr>
<td>HU-210, JWH-133 Cannabinoid Agonists</td>
<td>H3K9me3; Global levels</td>
<td>CB$_2$R- and CB$_2$R-expressing human glioma stem-like cells (U87MG and U873MG lines)</td>
<td>Induction of differentiation, inhibition of gliomagenesis</td>
<td>(31)</td>
</tr>
<tr>
<td>HU-210 Cannabinoid Agonist$^{+}$</td>
<td>miRNAs</td>
<td>Adolescent rat brain (entorhinal cortex)</td>
<td>Altered mRNA profile</td>
<td>(80)</td>
</tr>
</tbody>
</table>

CB$_2$R, type 1 cannabinoid receptor; CB$_2$R, type 2 cannabinoid receptor; HDAC3, histone deacetylase 3; H3K4me3, trimethylation of lysine 4 on histone H3; H3K9me2, dimethylation of lysine 9 on histone H3; H3K9me3, trimethylation of histone H3 on lysine 9; H3K27me3, trimethylation of histone H3 on lysine 27; H3K36me3, trimethylation of histone H3 on lysine 3; Met, methionine; mRNA, messenger RNA; miRNA, microRNA; NAc, nucleus accumbens; THC, Δ$^{9}$-tetrahydrocannabinol.

$^{+}$Cannabinoids have been shown to affect epigenetic regulation in brain or neurons.
Human Epigenetic Studies

Of the different components of the eCB system, several investigations have focused on the epigenetic regulation of the cannabinoid receptor type 1 gene (CNR1), which encodes the CB1R (Figure 3). Specific genomic elements of the CNR1 gene have been shown to interact with trans-acting factors, some of which are implicated in methylation of CpG sites in the DNA and histone posttranslational modifications (56–58). A few of these studies have revealed that CB1R expression is dysregulated in different pathological conditions and upon exposure to drugs of abuse. For example, CB1R expression is increased in peripheral blood lymphocytes of schizophrenic patients with cannabis abuse and is inversely correlated to methylation of the CNR1 promoter (Table 1) (59). However, that study had limitations in that most cannabis users also reported alcohol and cigarette use and were diagnosed with schizophrenia, making the direct delineation of any specific cannabinoid effect difficult. Nevertheless, CNR1 messenger RNA (mRNA) expression levels and promoter DNA methylation status detected in the blood were related to measures of cannabis craving, the severity of nicotine dependence, and severity of cannabis (and alcohol) consumption that suggest a relationship to brain function. As such, lymphocyte CNR1 DNA methylation and CNR1 mRNA expression could potentially serve as peripheral biological markers. Clearly, a greater number of studies are needed to replicate these findings and to establish causal relationships to fully understand the functional relevance of peripheral epigenetic disturbances to neurobiological alterations induced by drug use. Moreover, whether such associations are evident in cannabis users without other comorbid neuropsychiatric conditions is also important to address.

One of the first gene × environment epigenetic associations described with cannabis use relevant to psychiatric vulnerability involved the COMT gene and schizophrenia risk. COMT (encodes catechol-O-methyltransferase that metabolizes catecholamine neurotransmitters such as dopamine) has also long been implicated in substance use disorder. A well-known Valine 108/158 Methionine (Val108/158Met) COMT polymorphism increases COMT activity and thus levels of dopamine, which plays a critical role in reward, motivation, cognition, and addiction. This Val allele has generally been associated with increased substance use disorder (60,61) [but see meta-analysis in (62)]. Recently, Val108/158Met genotype interaction with COMT DNA methylation status in blood was associated with nondaily cannabis use, which was not observed in either daily users or nonusers. Thus, adolescents with the Met/Met genotype in combination with high rates of COMT promoter methylation were less likely to be high-frequent cannabis users than adolescents with the Val/Val or Val/Met genotype (63). Given that the status of COMT DNA methylation depended on the frequency of cannabis use in active using adolescents, it remains unanswered whether such epigenetic alterations persist long after these individuals stop using the drug.

It is evident that a complex relationship exists between genetic and epigenetic interactions, and the relationship between peripheral epigenetic marks and methylation status in the brain is still unknown. Despite the apparent associations of cannabis exposure with discrete molecular alterations in humans and the possibility to conduct studies on genetic associations, the specificity of the observed disturbances attributed to cannabis must be verified, especially in the light of potential polysubstance exposure, which is common in humans. In addition, cannabis consists of over 60 cannabinoids, one of which is THC, and cannabis preparations can largely differ in amounts of these various cannabinoids, typically confounding clinical studies. Another important limitation is that given the low incidence of cannabis-related mortality that would allow postmortem brain molecular analyses, most human epigenetic studies can only be conducted in the periphery of live subjects and thus their relationships with brain changes remain unclear. Nevertheless, the accumulating data indicate epigenetic disturbances in human subjects relevant to cannabis use disorders that would predict the potential for long-term molecular alterations.

Cannabinoid Animal Models and Epigenetic Factors

Animal models provide more controllable experimental strategies in which the protracted molecular consequences of long-term cannabinoid exposure can be better explored with regard to epigenetic mechanisms that could potentially maintain abnormal gene regulation and related behavioral disturbances. Such preclinical animal studies also facilitate the direct causal investigation of protracted effects in the brain as a consequence of developmental exposure to cannabinoid drugs. A number of early seminal animal studies demonstrated prenatal THC exposure on offspring behaviors and some suggested changes in gene expression (64,65), confirmed by subsequent investigations (66–67). More recent research efforts into the developmental effects of THC directly described epigenetic alterations germane to addiction disorders. These studies focused in large part on the nucleus accumbens (NAC), a critical neuroanatomical substrate underlying the pathophysiology of addiction (68–71). The CB1R is abundantly expressed on medium spiny neurons that represent the most abundant striatal cell type and constitute the differential output pathways (striatopallidal and striatonigral) that regulate specific behaviors. Interestingly, exposure to low- to moderate THC dosing paradigms has generally induced significant alterations of the dopaminergic D2 receptor (Drd2) and the opioid neuropeptide proenkephalin (Penk) genes (9,32,33,68), which are preferentially expressed on the striatopallidal neurons and have been linked with epigenetic impairments. The sensitivity of Drd2 and Penk to cannabis/THC exposure in both the human fetus and animal models is intriguing given the role of these genes in drug addiction vulnerability. Both human and animal postmortem studies
have revealed specific disturbances in the expression of the PENK and DRD2 genes in the NAc of subjects exposed to THC during either prenatal or adolescent developmental periods that persists into adulthood (32,33). Of the multiple epigenetic mechanisms, the regulation of histone modification is unique because methylation of distinct residues can have antagonistic effects on transcription (Figure 2C). Indeed, our previous studies revealed disturbances in the histone modification profile in the NAc of adult rats with prenatal THC exposure. These studies identified decreased levels of the trimethylation of lysine 4 on histone H3 (H3K4me3), a transcriptionally permissive mark, and increased levels of dimethylation of lysine 9 on histone H3 (H3K9me2), a repressive mark, as well as decreased RNA polymerase II association with the promoter and coding regions of the gene in the NAc (Table 1) (32). The combined epigenetic alterations were consistent with the observed reduction of the Drd2 gene expression and emphasize the enduring consequences of THC exposure following prenatal development. Similarly, persistent changes in repressive H3K9me2 and trimethylation of histone H3 on lysine 9 (H3K9me3) were observed at the Penk locus in the NAc of adult rats following adolescent THC exposure in line with enduring upregulation of Penk mRNA levels (33). These findings emphasize an altered epigenetic landscape within the adult brain directly as a consequence of developmental cannabinoid exposure.

There is also evidence that THC exposure can affect the regulation of histone modification in other cell and tissue types during development. In differentiating mouse lymph node cells, alterations in H3K4me3, H3K9me2, trimethylation of histone H3 on lysine 27 (H3K27me3), and trimethylation of histone H3 on lysine 36 (H3K36me3) have been associated with dysregulated ncRNAs and mRNA genes (34). In addition, THC treatment dose-dependently increased the expression of histone deacetylase 3 (HDAC3) in a human trophoblast cell line, indicating the possibility for cannabinoid exposure to affect placental development (72).

The studies discussed above highlight the long-term effects of cannabinoid exposure that influences the development of various cell and tissue types with functional and phenotypic consequences. Since these investigations so far have mainly been carried out at specific sets of candidate gene loci, rigorous future work will require comparisons between epigenomic and transcriptome alterations to address the mechanistic implications of these findings on the level of complex biological systems in different tissue types and their dynamic regulation throughout development.

MULTIGENERATIONAL EFFECTS OF CANNABIS

It has long been a subject of debates as to whether epigenetic disturbances that occur during the life span of an individual are reprogrammed across most of the genome from parent to offspring, thereby establishing a new epigenetic slate for the next generation. Such concepts have been challenged in recent years by findings in various disease states where epigenetic aberrations that influence disease risk were shown to be inherited through the germline from parent to child (27,73). More specifically, several cases of parent-child transmission regarding drugs of abuse have been published, describing both behavioral phenotypes and molecular disturbances in the offspring of parents that were exposed to drugs before mating [reviewed in (74)].

We have previously demonstrated that exposure of male and female adolescent rats before mating (germline exposure) leads to behavioral and molecular abnormalities in their unexposed offspring (11). Adult offspring of THC-exposed parents displayed increased work effort to self-administer heroin, with stereotyped behaviors during the period of acute heroin withdrawal. On the molecular level, parental THC exposure was associated with changes in the mRNA expression of cannabinoid, dopamine, and glutamatergic receptor genes in the striatum and altered synaptic plasticity in neurophysiological measures. In a more recent study and in line with the initial observations, DNA methylation disturbances were detected in the NAc of adult rats with parental germline THC exposure in an epigenome-scale investigation (15). The most significant finding was the identification of epigenetic alterations within an interaction network centered around the Dlg4 gene encoding postsynaptic density protein 95, a membrane associated guanylate kinase scaffolding protein located in neural postsynaptic densities involved in the regulation of dopamine-glutamate interactions. Psd-95 associates with the N-methyl-D-aspartate subtype of glutamate receptors and is required for synaptic plasticity associated with N-methyl-D-aspartate receptor function. A variety of genes involved in glutamatergic neurotransmission were also found to contain DNA methylation changes in the offspring of THC-exposed rats. Previously, epigenetic dysregulation of Dlg4 has been linked to abnormal glutamatergic transmission involved in morphine conditioning (75), consistent with the earlier observations of increased heroin self-administration in adult offspring with germline THC exposure (11). In other studies and in line with the above observations, adolescent female rats treated with the cannabinoid agonist WIN-55,212 before mating and pregnancy had progeny that exhibited increased morphine sensitivity (14,76). These findings demonstrate that germline cannabinoid exposure can impact offspring phenotype, can affect the molecular characteristics of the brain, and could possibly confer enhanced risk for addiction disorders.

Multigenerational epigenetic effects occur when an environmental trigger induces epigenetic changes that can be observed in at least one subsequent generation. The observations summarized above fit the classic concept of epigenetically inherited phenotypes. In-depth investigations are still needed to provide insights about epigenetic mechanisms underlying the transmission of cannabis effects through the germline. Moreover, important questions remain to be answered as to whether this represents a true transgenerational epigenetic transmission to subsequent generations (grandchildren and beyond) without direct germline exposure.

The eCB system plays important roles not only in the development of a variety of somatic cells and physiological systems but also in reproduction. It is known that both male and female reproductive tissues express CBRs and eCBs and that in male subjects, THC can disrupt gonadal functions (10,77). Studies on the impact of cannabinoids on epigenetic changes in male fertility have been conducted in Cnr1 null mutant mice that displayed higher histone retention in germ cells compared with the wild-type mice (78). In that study,
CB₂R expression was demonstrated to be necessary for spermiogenesis by controlling chromatin condensation in the developing sperm via the regulation of histone displacement during spermiogenesis, resulting in poor sperm quality. Adverse effects of cannabis use on the ovary of female subjects have also been found to present a higher risk of primary infertility due to anovulation. Even when marijuana-using women undergo in vitro fertilization treatment, they produce poor quality oocytes and lower pregnancy rates (79). The effects of cannabis on the oocyte epigenome that could potentially lead to multigenerational transmission remain to be explored. Specifically, subsequent studies are required to assess how possible epigenetic processes (e.g., DNA methylation) are involved in the transmission of cannabinoid effects from parent to offspring.

SUMMARY

Although still quite sparse in the number of studies and current mechanistic depth, there are solid scientific data that document protracted effects of cannabinoids on the brain as well as in other organs. Based on the current rapid growth in this scientific field, it is expected that significant developments in the near future will fill critical gaps of knowledge by focusing attention on long-term epigenetic processes and behavioral consequences of cannabis exposure.

The majority of addiction-related epigenetic neurobiological studies have targeted the adult brain. Even conceptually, very few studies have considered the potential lifelong or multigenerational epigenetic impact of cannabis. Although identifying mechanisms by which cannabis effects are maintained and transmitted is intriguing by itself, such explorations have potential far-reaching impact in the broader domain of developmental neurobiology, since the identified epigenetic processes will no doubt be fundamental to transmission of other environmental insults across generations that bear on psychiatric vulnerability.

The mechanistic links between epigenetic modifications and gene expression impairments will require rigorous comparisons between epigenomic and transcriptome alterations. The overlay of results from approaches like RNA sequencing, chromatin immunoprecipitation sequencing, and genome-scale DNA methylation studies in alignment to the genome will provide a unique potential to correlate epigenetic marks with the transcriptional regulation of neighboring genes. Moreover, the specific distribution and changes in 5-methylcytosine and 5-hydroxymethylcytosine (a demethylation intermediate) (Figure 2B) have not yet been studied in the context of cannabis and will likely be an interesting direction for in-depth mechanistic investigations. Importantly, direct causal relationships will be gained through the use of genomic editing tools to determine the impact of specific epigenetic disturbances in relation to gene expression. Providing causal links between gene expression impairments and specific behavioral phenotypes using in vivo gene manipulations offers important mechanistic value and the potential for developing targeted therapeutic solutions.

Overall, the integration of information garnered from clinical populations with data emerging from animal models will provide innovative insights to guide future translational studies and better inform clinical treatment and prevention strategies for the long-term impact of cannabis and even for the growing use of synthetic cannabinoids.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by grants from National Institutes of Health/National Institute on Drug Abuse Grant Nos. DA030359 and DA033860. The authors report no biomedical financial interests or potential conflicts of interest.

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Received Mar 5, 2015; revised Sep 1, 2015; accepted Sep 24, 2015.

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effects from parent to offspring.
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