Neuroepigenetics and addictive behaviors: where do we stand?

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Abstract

Substance use disorders involve long-term changes in the brain that lead to compulsive drug seeking, craving, and a high probability of relapse. Recent findings have highlighted the role of epigenetic regulations in controlling chromatin access and regulation of gene expression following exposure to drugs of abuse. In the present review, we focus on data investigating genome-wide epigenetic modifications in the brain of addicted patients or in rodent models exposed to drugs of abuse, with a particular focus on DNA methylation and histone modifications associated with transcriptional studies. We highlight critical factors for epigenomic studies in addiction. We discuss new findings related to psychostimulants, alcohol, opiate, nicotine and cannabinoids. We examine the possible transmission of these changes across generations. We highlight developing tools, specifically those that allow investigation of structural reorganization of the chromatin. These have the potential to increase our understanding of alteration of chromatin architecture at gene regulatory regions. Neuroepigenetic mechanisms involved in addictive behaviors could explain persistent phenotypic effects of drugs and, in particular, vulnerability to relapse.

Abbreviations

5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; CPP, conditioned place preference; DNAhm, DNA hydroxymethylation; DNAm, DNA methylation; DNMT, DNA

methyltransferase; DS, dorsal striatum; HDAC, histone deacetylase; MeCP2, methylated DNA-binding protein 2; miR, microRNAs; NAc, nucleus accumbens; PFC, prefrontal cortex; SUD, substance use disorder;

1-Introduction

Drug addiction or substance use disorder (SUD) is a complex brain disease involving long-term changes that lead to compulsive drug seeking, craving, and a high probability of relapse (Berridge, 2017; Volkow et al., 2016). Uncontrolled drug intake is associated with negative emotional states like anxiety when the drug is not available, and occurs despite individual and social negative consequences (Everitt and Robbins, 2005; Koob, 2009). The neuronal circuits involved in addictive behaviors are complex (Koob and Volkow, 2010), and imaging tools have identified disrupted brain structures in addicted humans that are implicated in addiction vulnerability (Volkow and Morales, 2015). Key structures include the mesolimbic dopaminergic system, with the ventral tegmental area (VTA) neurons projecting to the nucleus accumbens (NAc part of the ventral striatum). Other regions involved in motivational and emotional processes or in memory association such as the dorsal striatum (DS), prefrontal cortex (PFC), insula, extended amygdala and hippocampus (hipp), participate in this reward circuit. The rostromedial tegmental nucleus (RMTg) has been recently described to play a role in the control of the mesolimbic pathway (Bourdy and Barrot, 2012). Also, the lateral habenula is disrupted by drugs of abuse and proposed to be a hub in addictive responses (see Mathis & Kenny, in this special issue, and (Velasquez et al., 2014);). Only a proportion of individuals become addicted and this vulnerability, meaning the transition from recreational to uncontrolled and compulsive intake, is largely impacted by the nature of the drug, genetic factors, in addition to the developmental, social and the psychological context of the individual (EMCDDA, 2017; Juli and Juli, 2015; Kreek et al., 2005).

Knowledge of molecular mechanisms involved in behavioral adaptations observed following repeated drug exposure has greatly improved over the last decade. Modifications at the level of neuron connectivity signaling, dendritic spine morphology or synaptic plasticity (Robinson and Kolb, 1999; Ron and Jurd, 2005) have been described together with transcriptional regulations (reviewed in (Contet et al., 2004; McClung et al., 2005; Przewlocki, 2004; Rhodes and Crabbe, 2005; Russo et al., 2010; Spanagel and Heilig, 2005). Increasing evidence is now emerging that epigenetic modulations participate in vulnerability to addiction, as well as in the maintenance of behavioral adaptations induced by substance abuse. Epigenetic mechanisms involve chromatin modifications (chemical or conformational) that alter gene function without changing the DNA sequence. These mechanisms are often initial responses to a dynamic environment and regulate several processes including gene expression, DNA replication and repair, growth, cell cycle and, development. In the last years,

such dynamic modifications have been specifically studied in neurons, in relationship to neurodevelopmental, psychiatric and neurodegenerative disorders (Cholewa-Waclaw et al., 2016; Francelle et al., 2017; Nestler et al., 2016). As a result, the concept of neuroepigenetics emerged to specifically describe epigenetic regulations occurring in post-mitotic neurons as opposed to epigenetic mechanisms involved in developmental processes (Day and Sweatt, 2010). Several neuroepigenetic regulations occur throughout reward circuitry to regulate gene expression changes following exposure to drugs of abuse (Walker et al., 2015), which include addition of epigenetic marks on histone proteins or DNA sequence (**Figure 1** and see below). Additional modifications involve changes in non-coding RNA levels that impact gene expression and function and have been recently described in cocaine addiction (Smith and Kenny, 2018). They do play a critical role in synaptic plasticity underlying addiction and will not be detailed here (Mayfield, 2017; Smith and Kenny, 2018). As the epigenetic field is rapidly evolving, recent reviews dedicated to specific drugs like alcohol (Farris et al., 2015; Pandey et al., 2017), cannabinoids (Szutorisz and Hurd, 2017) or cocaine (Sadri-Vakili, 2015; Vaillancourt et al., 2017; Zwiller, 2015), and to a more global view of addiction (Nestler, 2014) have been published to highlight the importance of epigenetic dysregulations in addictive behaviors.

In the present review, we focus our attention on recent data interrogating global epigenetic modifications (genome-wide) in the brain following drugs of abuse in humans, non-human primates or rodent models, with a particular focus on DNA methylation and histone modifications, associated with transcriptional studies. We specifically highlight critical factors that should be considered when evaluating neuroepigenetics reprogramming. We discuss whether shared mechanisms or biomarkers have been identified within epigenetic programming following drug exposure. We highlight how developing tools, including investigation of structural reorganization of the chromatin, will increase our understanding of these molecular adaptations in addictive behaviors, that could explain persistent phenotypic effects of drugs and, in particular, vulnerability to relapse.

2-Critical factors for epigenome analysis in addiction

Recent studies in the addiction field have focused on epigenomic approaches, with the analysis of global changes at the level of chromatin for DNA methylation or histone modifications. These data are listed for psychostimulants (**Table 1**), alcohol (**Table 2**), opiate

(**Table 3**), nicotine or cannabinoid (**Table 4**), the latter still being scarce. Recent data are emerging from genome-wide analysis, using chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq). This allows quantification of the amount of immunoprecipitated fragmented chromatin for all genomic regions by high-throughput sequencing, using an antibody specific for a given targeted histone mark. Also, for global analysis of epigenetic dysregulations, a concomitant analysis of the gene expression profile represents a powerful tool to correlate the observed adaptations. Transcriptional analysis within brain reward circuitry has been widely performed in the field of addiction using either candidate approaches or microarray technologies (for reviews see (Contet et al., 2004; McClung et al., 2005; Przewlocki, 2004; Rhodes and Crabbe, 2005; Russo et al., 2010; Spanagel and Heilig, 2005). The emergence of high-throughput sequencing with the development of RNA-seq allows powerful transcriptional profiling. This method, based on the creation of cDNA libraries from isolated RNA, provides a reliable analysis of the transcriptome, at a higher base-pair resolution than classical microarray, and allows detection of non-coding RNAs, or alternative splice variants (**BOX**). We therefore also examined studies, which also provided transcriptomic analysis using RNA microarray or RNA-seq approaches, enabling to assess drug-induced epigenetic changes on gene expression programs. Specific results will be discussed below but several factors have to be considered when examining such genome-wide studies and we highlight the most critical ones here (**Figure 2**).

2.1-Animal models

Most of the studies investigating modifications in the brain have been performed in rodents, with the exception of a few ethanol studies, that examined human or rhesus macaque samples (see **Table 2**). Also, we have not included studies investigating epigenetic adaptations from human blood samples. Using rodent animals, one expects to obtain less variability compared with human studies performed on large cohorts of persons with distinct and often unknown histories of addiction. In contrast, various behavioral responses to drugs of abuse, like alcohol, are also observed in animal models within a homogenous population. This vulnerability may be partly explained by subtle environmental changes that can also induce epigenetic modifications. Also, differences may be identified when comparing drug effect on distinct strains at the level of behavior (Ayranci et al., 2015) or transcriptomic adaptations (Grice et al., 2007; Korostynski et al., 2007). Still, rodent models are very useful to study drug effects under more controlled conditions.

2.2.Diversity of paradigms

Among the rodent studies, it is interesting to note that various protocols have been developed, from acute drug treatment (A) to subchronic or chronic (C) administrations and sometimes following withdrawal periods (W). Doses, route of administration as well as duration of treatments vary extensively across studies making it difficult to describe a global modification scheme. Also, most studies analyzed passive drug administration with very few studies investigating voluntary consumption in rodent models (see below, (Baker-Andresen et al., 2015; Cadet et al., 2017; Fernandez-Castillo et al., 2012; Fonteneau et al., 2017; Freeman et al., 2010; Massart et al., 2015). When more studies are available, it will be useful to compare passive versus voluntary intake to evaluate possible transcriptomic or epigenetic signatures of voluntary consumption of drugs of abuse.

In SUD, increasing research to investigate mechanisms that could explain long term adaptations leading to relapse have been developed. Nevertheless, the neurobiological mechanisms involved in long-term drug abstinence are still poorly understood. Very few studies investigating whole-genome changes have focused their attention on abstinent conditions. Earlier studies have investigated drug abstinence at the transcriptome level (Kuntz-Melcavage et al., 2009; Spijker et al., 2004) and even fewer studies directly compared adaptations across drugs. Interestingly, in an open-ended approach using microarray, chronic morphine treatment revealed a collection of genes in the extended amygdala which represented promising candidates potentially involved in drug dependence and craving (Befort et al., 2008). Expression of these candidate genes were evaluated following four weeks of cessation of drug treatment (morphine, nicotine, THC and alcohol) and a common transcriptional signature of protracted abstinence was identified (Le Merrer et al., 2012). Interestingly, unlike other drugs, cocaine differentially altered some behavioral responses in abstinent conditions and, oppositely modified expression of several genes (Becker et al., 2017). These findings emphasize differences between cocaine addiction and addiction to other drugs, though highlighting commonalities between opiate, alcohol, THC and nicotine abuse. These data complete accumulating evidence that specific adaptations in terms of behavior, connectivity, morphology or transcription can occur depending on the type of drug and questioned the unitary theory of addiction proposing common mechanisms for dependence and relapse processes (Badiani et al., 2011; Ozburn et al., 2015). Altogether, while whole-genome approaches for epigenetic adaptations are still needed to explore this hypothesis, one

has to keep in mind that generalizing conclusions from these studies mostly obtained with psychostimulants may not be always pertinent for the other drugs of abuse.

In summary, time-dependent effects of drugs of abuse on transcriptional and epigenetic modifications represent a critical factor, as emotional deficits are still detectable after long periods of withdrawal (Goeldner et al., 2011). Several time-points should be included in future studies, and as proposed by Baker-Andersen (Baker-Andresen et al., 2015), including animals with a history of drug seeking will bring insight into the memory process altered by drug exposure. This will be particularly important to decipher specific mechanisms involved in states facilitating relapse vulnerability. In addition, the adolescent period represents a time when drug exposure increases the risk of addiction. There is limited research examining epigenetic changes in normal brain maturation during adolescence (Mychasiuk and Metz, 2016), but these data will further our understanding of these complex processes for a more complete comprehension of how the effects of drug exposure changes across time.

2.3-Brain structure or cell type targets

The complexity of the reward-circuit makes the comparison across studies challenging as targeted brain structures vary across studies. Interestingly, most epigenetic studies focused on the NAc and PFC (see **Tables**), which represent the structures where most initial transcriptomic studies were performed (Nestler, 2014). Among key reward-related structures, the NAc has been widely studied as the motivational center in reward related responses and is a critical structure for the initial rewarding effects of psychostimulants, whereas the PFC was investigated, primarily, for its role in goal-directed behavior and decision-making. Adaptations within the dorsal striatum (DS) have received far less attention than those occurring in the NAc, while changes in this structure would better inform epigenetic regulations in link with expression of compulsive drug-seeking traits, including relapse (Belin and Everitt, 2008). Brown and colleagues showed decreased expression of synaptic plasticity-associated genes in the DS of animals categorized as relapse-vulnerable (Brown et al., 2011), a regulation related to decreased expression of specific miRNA in subregions of the DS (Quinn et al., 2015). It seems critical to evaluate changes in homogenous structures with few different cell types as there is now accumulating evidence that epigenetic modifications may be specific to particular brain structures and even to cell-types. Even subtypes of medium spiny neurons of the NAc are differently affected by epigenetic modifications (Hamilton et al., 2018). Several techniques have been developed to enrich or isolate specific cells from brain tissue, including immunopanning, laser capture micro-dissection, fluorescence-activated

(FAC) sorting and magnetically labeled antibodies (see Holt and Olsen, 2016). The latter technique allows neuronal, astrocytic, and microglia cell populations to be sorted in adult rodent brain at a low cost. Distinguishing between neurons and glial cells, as recently performed using FAC sorting in human samples of heroin addicts, appears now to be critical for a deep analysis of epigenetic mechanisms (see below, §3.2 (Kozlenkov et al., 2017). Noteworthy, few whole-genome studies directly compared transcriptomic changes occurring across several structures following nicotine and methamphetamine (Mychasiuk et al., 2013) or alcohol (Mulligan et al., 2017) exposure (see **Tables 1-3**), but no specific or global epigenetic signatures were established.

2.4-Sex differences

Risk taking is often higher in males, but effects of many drugs appear more deleterious in females. Indeed, anxiety and depression are higher in females and these represent strong predictors of addiction. One study explored epigenetic changes in both sexes for DNAm following ethanol exposure in the human cortex (Wang et al., 2016a). Interestingly, no adaptations could be observed in females. Whether this was due to a low number of female individuals under study or really reflects a sex difference is not clear. Interestingly, the study by Engmann on 3D chromatin conformation showed a specific increase in *Auts2* gene only in male mice (see below § 6.1, (Engmann et al., 2017). More studies are needed to explore these aspects to better understand epigenetic mechanisms in the future and clarify how gene expression influences individual heterogeneity in vulnerability to addiction.

3-Changes in DNA methylation and hydroxymethylation

DNA methylation (DNAm) and hydroxymethylation (DNAhm) have been predominantly studied in the mammalian developing brain and described as dynamic processes, as they play a critical role in the establishment and maintenance of cell identity (Bogdanovic and Lister, 2017). DNA methylation corresponds to the addition of a methyl group, usually on the 5' position of the carbon of the pyrimidine ring of cytosine, and occurs at dinucleotides CpG and CpH (H=A/C/T) (see **Figure 1**) (Kinde et al., 2015; Lister et al., 2013). This modification (5-mC), when occurring in the promoter region, is mostly associated with transcriptional repression and is catalyzed by DNA methyltransferases involved in the maintenance of methylation (DNMT1) or in de novo methylation (DNMT3 a and b). Demethylation processes are conducted by ten-eleven translocation enzyme which catalyzes the hydroxylation of the methylated cytosine (5hmC). Other forms are also produced, with 5-

formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are processed by DNA repair mechanisms and further generate unmethylated cytosine (Auclair and Weber, 2012). In contrast, hydroxymethylation in intragenic regions is associated with higher gene expression (Guibert and Weber, 2013; Kato and Iwamoto, 2014). Proteins with methylated DNA binding domain (MBD) play a critical role in DNA methylation, with the protein methyl CpG-binding protein 2 (MeCP2) being one critical reader of DNA methylation in the brain (Kinde et al., 2015). MeCP2 protein level is increased by cocaine and contributes to behavioral responses to psychostimulants (Cassel et al., 2006; Deng et al., 2010; Im et al., 2010). However, additional studies are needed to better understand the implications of this process. Methods for comprehensive analysis of DNA methylation and hydroxymethylation include bisulfite sequencing, or collection of methylated, hydroxymethylated, or unmethylated DNA by specific binding proteins, antibodies, or restriction enzymes, followed by sequencing or microarray analysis. These techniques allow measurement of DNA methylation at a single-base resolution (see **BOX**).

3.1-Correlation of DNAm with transcriptional adaptations

The possible correlation of DNAm changes induced by different drugs of abuse and gene expression at a genome-wide scale has been examined in a number of studies. In one, psychostimulant exposure induced differentially methylated regulations which were correlated with gene expression adaptations. In a study examining the effect of cocaine self-administration, combination of whole-genome and candidate approaches allowed identifying DNAm regulations in the NAc, which were partly negatively correlated with gene expression changes (Massart et al., 2015). Analysis of 5hmC (hMeDIP-Seq) in the NAc following a model of compulsive methamphetamine self-administration showed similar correlation (Cadet et al., 2017). Hydroxymethylation peak changes were found in intergenic sites while only few modifications were present at transcription start sites, exons and, introns. Interestingly, potassium channel genes with increased DNA hydroxymethylation peaks at intergenic sites were associated with an increased expression of potassium channel coding genes in exposed but non-compulsive rats (Cadet et al., 2017). In another study using methylome analysis in the PFC following self-administration of cocaine in rats, the authors have revealed that increased DNAm were inversely correlated to transcriptional activation when methylation occurred in the gene promoter. Interestingly, this was not observed when methylation took place inside gene bodies (Fonteneau et al., 2017), where a strong variation was observed in the tested genes with both up- or down- regulations associated with hypermethylation. Alternative

promoters in gene bodies or the presence of alternative splice sites could explain this observation. Also, similar methylation levels can occur in active and inactive gene bodies across brain tissue (Aran et al., 2011). A better understanding of these modifications will be provided with a more complete genome annotation and improved description of alternative promotor or splice sites. Others have described more mixed results in a cocaine self-administration paradigm, with both validation of transcriptional regulation associated with DNAm and non-regulated transcripts (Baker-Andresen et al., 2015). Whether these correlations are specific to the type of drugs is not clear. Indeed, following alcohol exposure, Wang et al. investigated DNAm changes in PFC postmortem samples from individuals with alcohol use disorders (Wang et al., 2016a). They found 1812 differentially methylated CpGs among which the majority were hypermethylated (1201 CpGs), and preferentially found in promotor regions and gene bodies. When analyzing these data together with previous transcriptomic data from the same samples (Zhang et al., 2014), the authors did not observe any correlation between DNAm changes and gene expression. The authors argue that this could be due to single nucleotide polymorphism on DNAm affecting gene expression. A similar finding with no correlation between DNAm and gene expression was obtained in a transgenerational study in rats using a binge alcohol protocol at adolescence (see §5.2, (Asimes et al., 2017). One can-not exclude the fact that the complexity of data in genome-wide analysis and the accuracy of genome annotation make bioinformatic analysis difficult for such correlations. Also, double analysis of DNAm and hydroxymethylation in future studies may help to clarify these aspects. Together, these data indicate more complex mechanisms for DNAm processes than initially described, which may specifically differ depending on the methylated gene loci or the drug abused.

3.2-Cellular specificity of DNAm

In a global DNA methylation profiling (DNAm microarray) of orbito frontal cortex (OFC) from heroin addicts who died of overdose, the authors investigated heroin effects on DNAm specifically in neuronal nuclei separated by FAC sorting (Kozlenkov et al., 2017). Hypermethylated regions were preferentially found in exons and gene bodies and mostly depleted from promoter regions, in genes involved in synaptic plasticity. In contrast, hypomethylated regions were preferentially found in promoter regions. To complete their analysis, they performed H3K27Ac ChIP-seq and showed that only hypomethylated regions were enriched in enhancers, at both distal (putative active enhancers) and proximal (active promoters) regions from the transcription start site, while hypermethylated regions were depleted. Investigating modification specifically in neurons reveals enhanced

hypermethylated gene regions in genes enriched preferentially in glutamatergic, but not GABAergic, neurons. Altogether, these results are in accordance with reduced glutamatergic transmission in the frontal cortex observed following drug exposure and particularly highlight that DNA methylation changes in neurons are specific to targeted gene regions. Other studies have recently analyzed transcriptomic profiling using RNA-seq in targeted cell-types. These studies were conducted in rodent paradigms with prolonged nicotine exposure, using laser dissected *Pomc* neurons, known to be involved in nicotine-anorectic effects (Silva et al., 2016), or SNc neurons, implicated in Parkinson disease and expressing high levels of nicotinic acetylcholine receptors (Henley et al., 2013). A recent study targeted microglial cells isolated from the prefrontal cortex of alcohol exposed mice, a population of cells involved in the neuroinflammatory response of alcohol (McCarthy et al., 2018). The results revealed subtle changes in gene expression following nicotine and a distinctive microglial gene expression signature for neuroimmune responses related to alcohol consumption. Targeting specific cell-types demonstrated the critical importance of studying DNAm changes in a highly homogenous cell population. Altogether, further studies dissecting cell-type specificity for epigenetic regulations should focus on more precise mechanisms involved in adaptations following drug abuse. Such approaches may encounter difficulties due to limitation in sorting cell-types or in DNA amount needed from specific brain structures. Nevertheless, technical advances are emerging very rapidly with approaches conducted with limited DNA amount or performing single-cell epigenome sequencing (Farlik et al., 2015; Wen and Tang, 2018), which will surely open more precise investigation of these neuroadaptations.

3.3-Dynamics of DNAm changes

Few studies have investigated the dynamics of DNA modifications at the transcriptomic or epigenetic levels. In the NAc of rats self-administering cocaine over 10 days, DNAm changes were examined either a day following the treatment (short) or 3 weeks after (prolonged withdrawal). The results revealed stable changes in DNAm at the two time-points illustrating a persistent adaptation. Interestingly, several modifications were time-dependent, indicating a more dynamic process associated with DNAm in link with behavioral adaptations induced by cocaine (Massart et al., 2015). Using a similar cocaine self-administration model in rats, others have compared gene expression profiles (RNA microarray) following 1, 10 or 100 days of withdrawal (Freeman et al., 2010). Their analysis also revealed complex adaptations, with several categories of time-dependent changes in gene expression in NAc and PFC, including persistent or unstable changes throughout withdrawal. In an elegant study investigating the effect of cocaine at several steps of dependence in a

mouse model of self-administration, the authors questioned the implication of adaptations from simple drug exposure versus learned cocaine-seeking (Baker-Andresen et al., 2015). The authors used methyl-binding protein immunoprecipitation followed by high throughput sequencing (MBD-seq) to measure DNAm in isolated neurons of mouse PFC. DNAm patterns were compared after acute and prolonged withdrawal or in a situation of relapse. Passive cocaine exposure and cocaine self-administration produced distinct patterns of 5mC enrichment. Persistent methylations were observed across the time-points, mostly hypermethylation, which were embedded within genes or located distal. Hypermethylation induced by cocaine is in line with increased expression of *DNMT3A* and *3B* observed following repeated administration (Anier et al., 2010; LaPlant et al., 2010; Pol Bodetto et al., 2013). Also, some methylation profiles were specific to mice subjected to 3 weeks of abstinence following cocaine self-administration but not in animals following one day of withdrawal or in yoked cocaine controls (Baker-Andresen et al., 2015). These data highlight the complexity of dynamic changes in DNAm following drug exposure, with implications of changes related to withdrawal or to learning processes with the maintenance of cocaine-related memories. Further studies investigating such time-dependent changes in DNAm are needed to clarify epigenetic adaptations underlying the cognitive adaptations that lead to addictive behaviors. In particular, exploring drug-seeking and relapse represent a major challenge to understanding long-term process observed in human addiction. Whether these adaptations are specific to psychostimulants or share common mechanisms across drugs is still an open question. In addition to methylation, hydroxymethylation also plays a major role in this dynamic process (Bachman et al., 2014), and this adds an additional level of complexity to DNAm mechanisms in the context of SUD.

3.4-DNAm at splicing sites

Recent studies have highlighted that DNAm may occur at specific splicing sites in the gene and therefore not directly impact gene expression levels, but rather the expression of distinct variants. Consequently, this may differentially modulate synaptic plasticity or signaling process and thus have specific phenotypic impacts. In the study by (Baker-Andresen et al., 2015) (see above), 12 of 15 persistent gene-associated DMRs were located within intronic regions or non-coding loci, whereas principal promoter regions and exons were relatively devoid of changes. These DNAm changes induced by cocaine self-administration were able to modify alternative splicing and therefore expression of isoforms. Therefore, they demonstrated that the absence of an overall change in a gene expression did not exclude alteration of splice variant expression. In Nestler's group, using ChIP and RNA-seq in the

NAc of cocaine-treated mice, they established chromatin and transcriptional profiles with the identification, for the first time, of the transcription factor *E2F3* as a regulator of cocaine-induced gene expression and alternative splicing (Feng et al., 2014). In a follow up experiment, they assayed the effects of NAc overexpression of *E2F3* isoforms on cocaine behavioral response and transcriptomic profiling (RNA-seq) (Cates et al., 2017). Interestingly, overexpression of *E2F3a* was sufficient to mediate cocaine-induced locomotor sensitization and cocaine CPP, and the knockdown prevented these responses. *E2F3a* overexpression induced differentially expressed genes and differential alternative splicing events in a similar manner as cocaine, and both cocaine and *E2F3a* overexpression increased *E2F3* binding at consensus sequences near alternative splicing sites (Cates et al., 2017). These findings reveal a crucial role for *E2F3a* as a key regulator of cocaine-elicited molecular actions with both transcriptional and splicing profile changes leading to behavioral adaptations to drugs of abuse. In a study analyzing previously published data using an innovative bioinformatics approach to model association between splicing sites and histone marks (Feng et al., 2014), Hu et al found two marks (H3K36me3 and H3K4me1) with the strongest association with alternative splicing (Hu et al., 2017), indicating a major role for histone methylation in this process. In another study from the same group, repeated cocaine administration effects on DNA hydroxymethylation (5hmC-seq) in the same brain structure revealed a majority of 5hmC distributed in gene bodies and intergenic regions (Feng et al., 2015). Combining these results with analysis of both Chip-seq (H3K27Ac and H3K4Me1) and RNA-seq, the authors observed 24 genes showing an increase in both 5hmC and mRNA 24 hours after the last cocaine injection. They demonstrated that 5hmC dynamic modulation correlated with putative enhancers. Moreover, 5hmC regulations were found at exon boundaries which indicated the involvement of 5hmC at splicing sites. Consequently, hydroxymethylation correlated with splicing isoforms up-regulation. Together, these results indicate that 5hmC alterations represent a mechanism inducing long lasting changes and play a major role in cocaine-induced adaptations. This may potentially be extended to other drugs of abuse.

Interestingly, altered splicing sites have been studied in neurodevelopmental disorders such as schizophrenia or depression. For example, an alternative splicing of *GAD1* encoding glutamic acid decarboxylase was altered by DNA methylation at a CpG island close to the putative promoter of *GAD1* in cortex and hippocampus of patients with schizophrenia (Tao et al., 2017). Whether this adaptation was specific to neurons is still not clear. Another study revealed potential alteration of splicing sites for the glutamate ionotropic kainate receptor *GRIK2* induced by hypomethylation at intronic sites in isolated astrocytes cells from PFC of

individuals who died during a depressive episode (Nagy et al., 2015). Taken together, it underlines possible general mechanisms involved in psychiatric disorders, that would be common with addiction.

In summary, a combined analysis of methylome (both methylation and hydroxymethylation) and transcriptome will be informative in assessing splice variants expression and understanding time-dependent methylation changes following drug exposure. The current studies have highlighted a dynamic process of methylation and hydroxymethylation following psychostimulant exposure. Whether similar conclusion can be proposed for the other drugs of abuse has still not been explored. The human studies are confronted with the constraint of analysis of DNAm at a unique time point in postmortem tissue (Cecil et al., 2016b). Whether extrapolation of data from studies examining DNA methylation in blood samples would be feasible is still questionable.

4-Modifications of Histones

Another epigenetic mechanism that influences chromatin structure and thus the interaction between DNA and histones is the direct occurrence of modifications at the level of the N-terminal part of the histone tail (Walker et al., 2015). For example, acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation modulate in a reversible manner the degree of compaction of the chromatin. Specific enzymes are implicated in these processes to establish the marks (writers) or erase them (erasers). Histone acetyltransferases and histone deacetylases (HDAC) target specific histone residues for acetylation and deacetylation, respectively. Histone methyltransferases and demethylase act in a similar fashion for methyl groups. This represents a dynamic process. Typically, amino-acid acetylation promotes an open chromatin state associated with transcriptional activation. Histone methylations, usually on Lys and Arg, lead to activation or repression depending on the targeted residue. Methylated Lys 9 of Histone 3 (H3K9me) and H3K27me are associated with transcription repression, while H3K4me enhances transcription. In addition, the location of these modifications, at the promoter or regulatory regions, increases the complexity level of these regulations. These posttranslational mechanisms in response to psychostimulants have been widely studied using candidate gene approaches (Rogge and Wood, 2013; Sadri-Vakili, 2015; Walker et al., 2015). In addition, new tools allow studying the chromatin accessibility with DNaseI-Seq/ATAC-Seq to profile open chromatin or the spatial organization with chromosome conformation capture (3C)-based techniques (see **BOX**). Studies on DNAm and hydroxymethylation pointed to modifications at the level of enhancers, suggesting that their

activity may be modulated by drugs of abuse by DNA methylation processes. Studying modifications of histones, in particular the ones associated with enhancers, will provide insights into the role of DNA methylation.

4.1-Histone acetylations

Most studies examining histone modifications focused on the effects of passive cocaine exposure (see **Table 1**). A first study from Nestler's group has examined the role of histone modification in addictive behaviors and demonstrated that chronic cocaine treatment induced histone deacetylase 5 (*HDAC5*) gene expression in the NAc (Renthal et al., 2007). This enzyme usually represses gene expression by deacetylating histones. Interestingly, overexpression of *HDAC5* in the NAc attenuated cocaine CPP while *HDAC5* deficient mice showed a behavioral enhancement. This epigenetic enzyme has also been implicated in methamphetamine craving in the DS (Li et al., 2017; Li et al., 2015). The genomic effect of repeated cocaine in the NAc was further studied with chromatin immunoprecipitation coupled with promoter microarray analysis (ChIP-chip) for acetylated and methylated histone (Renthal et al., 2009). Cocaine increased both acetylation and methylation at many more genes than it induced decreases. There was no systematic correlation between chromatin modifications and gene expression. The results also revealed concomitant hyperacetylation of H3 and H4 in very few genes, suggesting that the two marks play distinct roles (Renthal et al., 2009). The authors used ChIP for ΔFosB to identify direct targets for this transcription factor known to be regulated by cocaine (Renthal et al., 2009). They identified a particularly interesting gene family, the sirtuins or Sirt (silent information regulator of transcription) which are histone deacetylases whose function in the nervous system is still not clearly understood. The authors identified a significant enrichment of the transcription factor on the *Sirt2* promoter together with an increase of acetylated H3, associated with an increase of *Sirt2* transcript in the NAc. The same finding was obtained for *Sirt1*. Also, only chronic cocaine was able to increase both *Sirt1* and *Sirt2* activity in the NAc, compared to a single drug administration. Interestingly, pharmacological activation and inhibition of sirtuins were able to respectively enhance and diminish cocaine CPP as well as electrical excitability of NAc neurons (Renthal et al., 2009). In addition, a dramatic reduction in cocaine self-administration was observed following inhibition of sirtuins. Altogether, these findings implicate sirtuins in regulating behavioral effects of cocaine but whether this effect is due to histone modification or to sirt modulation of signaling process is not fully established. With a similar protocol in mice, a ChIp-seq approach was performed and the authors demonstrated that *Sirt1* activation induced the

transcription factor forkhead box O3 in the NAc. Interestingly, the overexpression of this factor in the NAc enhanced cocaine CPP (Ferguson et al., 2015). Altogether, these studies highlighted a role for sirtuins in cocaine-induced adaptations at the molecular and behavioral levels but the precise mechanism involved is still not fully established. Interestingly, *Sirt1* overexpression in the NAc increased cocaine or morphine rewarding effects (Ferguson et al., 2013), suggesting a role for the sirtuin family across drugs of abuse. Whether similar implication of these HDAC is also observed in self-administration paradigm would be critical to better understand underlying adaptations for long term drug exposure and relapse events. Noticeably, sirtuins have been implicated in depressive-like state, a disorder often associated with SUD (Kim et al., 2016).

Interestingly, histone acetylation in the striatum was also observed in a model of methamphetamine exposure using a combination of microarray and ChIP-seq analysis (Cadet et al., 2013). Particularly, methamphetamine caused significant increases in H4K5Ac binding which correlated with levels of gene expression. Many regulated pathways were identified such as cell death and survival and nucleic acid metabolism. As in Martin et al. (Martin et al., 2012), the authors found an upregulation of several transcription factors like *cFos*, *Egr1* and *Egr2*. In addition, their findings revealed that acute and chronic administration of methamphetamine induced differential regulations in striatal gene expression, with more increased gene expression after an acute injection and mostly decreased gene expression following chronic treatment. Also, acute treatment induced additional H4K5Ac binding sites in more genes than in the chronic situation. These observations suggest that epigenetic adaptations to single drug exposure may be distinct from adaptions to long-term exposure, which may have triggered other epigenetic factors to differentially impact gene expression. This highlights potential specific epigenetic modifications in situation of chronic treatment with psychostimulant and, moreover, in conditions involving drug-seeking associated learning process.

4.1-Histone methylations

Histone methylations seem to play a major role in cocaine effects, with activation or repression of gene expression depending on location and number of methyl groups involved. Nestler's group investigated the relationship between two histone lysine dimethyltransferase G9a and GLP (G9a-like protein) and cocaine treatment. The authors observed a down regulation of both proteins in the NAc, induced by chronic cocaine exposure, which was associated with a decrease of the repressive mark H3K9me2 (Maze et al., 2010). Using

genetic animal model and viral approaches, the authors demonstrated that G9a down regulation was able to modify neuron morphology in the NAc and enhance preference for cocaine. However, G9a overexpression in the NAc shell of rats increased H3K9me2, which surprisingly enhanced motivation for cocaine self-administration associated with increased anxiety (Anderson et al., 2018). Repeated cocaine decreased both H3K9me2 and G9a in the NAc of mice and, infusions of an HDAC inhibitor increased global levels of histone acetylation but also, of repressive histone methylation and G9A expression, illustrating cross talk among different types of histone modifications in the adult brain (Kennedy et al., 2013). Also, in a study modulating G9a expression specifically in *Drd1* or *Drd2* expressing neurons using genetic models or overexpression, distinct behavioral effects in response to cocaine were highlighted (Maze et al., 2014). In particular, selective deletion of G9a in *Drd2* neurons resulted in the unsilencing of transcriptional programs normally specific to *Drd1* neurons, coupled with acquisition of *Drd1*-associated projection and electrophysiological properties. Therefore, the authors proposed a new role for G9a in contributing to neuronal subtype identity. In another study from the same group, H3K9me3 was shown to be altered by repeated cocaine in the NAc (Maze et al., 2011). ChIp-seq analysis revealed that H3K9me3 was predominantly within intergenic regions and at repetitive genomic sequences. Similar finding was described following morphine treatment, with a specific decrease of G9a associated with diminished levels of H3K9me2 in the NAc mostly in intergenic regions and repetitive sequences (Sun et al., 2012). A ChIP-seq analysis was performed to further identify targets of G9a and, data confirmed previously described genes associated with morphine effects as well as novel targets, including glutamatergic signaling genes like *Grin2a*, *Grip1*, *Grm5*, and *Grm8.* Together, these findings suggest a critical role for histone methyltransferase, whose control of basal patterns of gene expression is altered by exposure to cocaine and morphine.

Interestingly, a recent study investigated the role of histone Arg (R) methylation in cocaine action and showed a decreased expression of protein-R-methyltransferase-6 (PRMT6) and its associated histone mark (H3R2me2a) in the NAc of mice or rats exposed to cocaine or in human addicts (Damez-Werno et al., 2016). This down-regulation is specifically observed in *Drd2* expressing neurons, and opposite in Drd1. Using ChIP-seq, they identified Src kinase signaling inhibitor 1 (Srcin1) as a target for reduced H3R2me2 binding, highlighting the effect of cocaine on another histone mark.

Altogether, histone acetylation and methylation are playing an important role in drug adaptations in the NAc, however, more studies are necessary to propose a molecular mechanism for such adaptations, particularly with distinct drugs of abuse.

5-Epigenetic transmission

Exposure to drugs of abuse during a sensitive period for brain development may be critical for epigenetic changes as they could be exacerbated later on in the adulthood or even become transgenerational (Bale, 2015). Such studies are highlighted in the tables (see ***** in **Tables**) and some of them discussed below.

5.1-Gestational exposure

Genome-wide approaches have revealed that in utero drug exposure could induce epigenetic modifications. Most of these focus on **nicotine** effects as numerous studies showed fetal brain development alterations due to maternal smoking (Banderali et al., 2015). Transcriptomic analysis was performed on offspring cortical samples from mice exposed to nicotine (200µg/ml) from parental mating to weaning, followed by a three-month withdrawal (Jung et al., 2016). The authors identified *Ash21*, a gene involved in histone methylation, as the most up-regulated transcripts by nicotine exposure during development. They further examined genome-wide changes in H3K4me3 by ChIP-seq analysis and identified most alterations induced by developmental nicotine exposure at promoter regions of genes involved in glutamate neurotransmission. Interestingly, knockdown of Ash2l abolished nicotine-mediated alterations of dendritic complexity and decreased nicotine-dependent changes in passive avoidance behavior. These data highlighted Ash2l, forming a complex together with the transcription factor *Mef2c*, as critical targets for nicotine to alter neuron morphology and alter persistent behavior during development. Altogether, these genome-wide approaches point to a novel mechanism of gene regulation during brain development involving H3K4me3 epigenetic mark.

In a study examining the impact of e-cigarette smoke exposure during early life development (e-cigarette aerosol, 3 h/day; 5 day/week until postnatal day 4-6, with or without 13-16 mg/ml nicotine), the authors analyzed gene expression alterations using RNA-seq in the mouse offspring frontal cortex (Lauterstein et al., 2016). They described pathways with common gene networks related to cancer, organismal injury and abnormalities, and gastrointestinal disease. Interestingly, sex-dependent transcriptional changes were observed

following aerosol exposure, with or without nicotine. In another study examining maternal nicotine exposure (nicotine in drinking water during gestation, 200µg/ml), the authors examined transcriptional profiling (RNA-seq) on laser dissected *Pomc* neurons from the offspring (Silva et al., 2016). This approach revealed one consistent change, the upregulation of Gm15851, a lncRNA of yet unidentified function. In a global DNA methylation profiling (DNAm microarray) of human dorsolateral prefrontal cortex of nicotine exposed fetuses, the authors investigated the role of DNAm in adaptations to nicotine effects and gestational age (Chatterton et al., 2017). Two hypomethylated regions within *SDHAP3* (involved in mitochondrial membrane function) and *GNA15* (encodes a G protein subunit) promoters have been identified specifically in exposed -fetuses. Interestingly, the authors observed an increase of *SDHAP3* transcript only in exposed -fetuses between early and late development. A similar finding was noted for *GNA15*, with a larger increase in exposed versus unexposed -fetuses. These data suggest that maternal nicotine exposure induces both gene expression and DNA methylation modifications during cortical development (Chatterton et al., 2017). Interestingly, modification of the differentially methylated region (DMR) levels of these two genes has been also observed in autism and schizophrenia studies in cerebellum and cortical areas (Ladd-Acosta et al., 2014; Nardone et al., 2014; Wockner et al., 2015). Altogether, this highlight a link between DNA methylation and neurodevelopmental diseases, and such implication may also apply to SUD.

Prenatal **ethanol** effects have been examined in rats using RNA microarray (Middleton et al., 2012) or RNA-seq (Ignacio et al., 2014), and specific genes or miRNA have been identified in amygdala and NAc. Interestingly, these regulations involving signaling pathways could participate in the social motivation deficits seen in adolescent rats produced by prenatal ethanol, an effect that was reversed by a social enrichment in the offspring. Another study using genome-wide analysis of gene expression in the mouse hippocampus revealed altered expression of 23 genes and 3 miRNAs in a gestational ethanol-exposure model (Marjonen et al., 2015), including MiR138-2 also detected in the previous study, but with an opposite regulation. These distinct results may reflect differences in duration and timing of exposure. Interestingly, the authors identified a Histone cluster 1 H2ai, which was down regulated by ethanol exposure, indicative of altered-chromatin conformation by alcohol, across generation.

5.2-Transgenerational inheritance

Transgenerational inheritance, reflecting transmission to unexposed offspring, has also been recently explored in the addiction field. Initial studies investigated candidate genes following alcohol, morphine or cocaine exposure. For example, Pomc gene hypermethylation caused by fetal alcohol exposure was transmitted to offspring through male germline (Govorko et al., 2012). Analysis of the progeny of adolescent female rats exposed to morphine showed an increase of D2 and kappa opioid receptor expression in two generations of mice, suggesting epigenetic adaptations (Byrnes et al., 2013). In another study, reduced cocaine intake was observed specifically in male offspring of cocaine-experienced males, coupled with an increased association of acetylated histone H3 (H3K9K14ac2) with BDNF promoters (Vassoler et al., 2013). This epigenetic adaptation was associated with increased baseline anxiety in these animals, which was unaltered by subsequent cocaine exposure (White et al., 2016).

More recent studies explored epigenetic inheritance using genome-wide approaches to better investigate molecular targets or pathways modulated in progeny. In order to evaluate the impact of smoking marijuana on subsequent offspring, one study examined the effects of parental THC exposure (during rat adolescence) on DNA methylation in the NAc of adult F1 unexposed rats using a bisulfite-sequencing approach. The authors identified 406 hypermethylated and 621 hypomethylated DMRs across the genome. Interestingly, the majority of DMRs were detected in gene bodies and downstream of transcriptional start sites, particularly within genes involved in the glutamatergic synaptic regulation (Watson et al., 2015). The DMR-associated genes exhibited altered mRNA expression in the NAc. A gene network centered on *Dlg4* coding for PSD95, a scaffolding protein involved in synaptic plasticity was identified, together with genes involved in glutamatergic neurotransmission. *Dlg4* gene is regulated by epigenetic factors in aging and neurodegenerative diseases like Alzheimer's disease, Huntington's disease and schizophrenia (Bustos et al., 2017). Notably, PSD95 plays a major role in the formation of synapses during specific time points of neurodevelopment and regulates synaptic function that influences behavioral phenotypes in SCZ (Coley and Gao, 2018). Alteration of DNAm of *Dlg4* by parental THC exposure could therefore be associated with an increased risk of schizophrenia in cannabis consumers (Hudson et al., 2018). In a similar approach, other authors have analyzed the effect of parental methamphetamine exposure on hippocampal samples of offspring using DNAm microarray targeting annotated CpG Islands and promoter regions (Itzhak et al., 2015). 545 CpG islands and 156 promoter region hypermethylated DMRs induced by in utero methamphetamine

exposure have been identified. Gene ontology analysis in the DMR highlighted “cerebral cortex GABAergic interneuron differentiation” for hypermethylated DMRs and “embryonic development” for hypomethylated ones. These results suggested that the observed phenotypes in F1 generation, including enhanced response to cocaine-conditioned reward and hyperlocomotion, and reduced fear conditioning, could be the result of abnormal brain development.

Another study investigated modification of DNAm profile (Bis-Seq) in hypothalamus of unexposed offspring of rats, whose parents were exposed using a binge alcohol protocol at adolescence (Asimes et al., 2017). The authors showed altered epigenetic modifications in these naïve rats which varied depending on parental exposure. Indeed, differentially methylated cytosines were distinct between offspring, depending on which parent was exposed to ethanol. In addition, the highest number of hypermethylated and hypomethylated regions identified was observed when both parents were exposed to ethanol. Finally, less is known about morphine exposure on offspring and only one study described transcriptomic adaptations (RNA-seq) using maternal morphine exposure (Vassoler et al., 2017). The authors mainly observed altered gene expression in the NAc for genes specifically involved in synaptic plasticity and neural development associated with a higher resistance to self-administer morphine in the offspring.

In summary, all these studies highlight that prenatal exposure to drugs of abuse alters neural development and synaptic plasticity, as well as phenotypic behavioral responses. The transmission to the offspring seems to involve epigenetic modifications, but the mechanistic process of these regulations at the chromatin level still needs to be further addressed, including with comparison to transcriptomic profiling. Investigation of the persistence of these regulations by examining regulations in subsequent generations (F2 and F3) would be helpful for a more comprehensive interpretation of these mechanisms. Human studies have examined regulation in blood samples (Cecil et al., 2016a; Markunas et al., 2014) for alcohol or nicotine early exposure and highlighted epigenetic changes but direct correlations with modifications occurring in brain structures may be difficult to establish.

6-Developping tools for neuroepigenetic studies

6.1-Chromatin 3D structure and psychostimulant addiction

More recent studies have used sophisticated approaches to decipher the architecture of the chromatin in response to drugs of abuse. One study raised the question of the alteration

of 3D architecture of chromatin induced by cocaine treatment, at genes showing epigenetic changes (Engmann et al., 2017). Using FAC sorting and viral mediated approaches, the authors found that *Auts2*, a gene linked to cognitive disorders, was enriched in *Drd2* medium spiny neurons in mice. An increased expression was also detected in cocaine-addict post-mortem NAc samples. In mouse NAc, using chromosome conformation capture-on-chip (4C-seq) investigating one specific locus vs all loci, the authors found that chromatin looping connected *Auts2* predominantly with a brain-specific calcium binding gene (*Caln1*) in basal conditions, an interaction that was disrupted following chronic cocaine treatment. Using bisulfite sequencing, they showed increased DNAm at the vicinity of *Auts2* interaction site. They further manipulated DNAm using CRISPR-genome editing in Neuro2a cells, targeting DNMT activity at the interaction site between *Auts2* and *Caln1*. This brought the evidence that DNAm alteration on *Auts2* gene could modify gene expression of a distant chromatin interaction partner, *Caln1* (Engmann et al., 2017). Even if this was shown in vitro due to current technical limitation in vivo, it demonstrated a causality between interaction and expression. Future technical improvement will certainly allow testing this mechanism directly in brain structures. These new findings provide novel insight for explaining the impact of epigenetic modifications on gene expression. Modification of expression in response to drugs may involve alteration of the 3D structure of chromatin at gene regulatory regions.

6.2-Epigenetic-based therapeutic interventions

Epigenetic modifications have been proposed as potential biomarkers for SUD (Cecil et al., 2016b). DNAm patterns in human blood cells may be used to identify vulnerability towards addiction in individuals. Hypermethylation in the 3'-protein-phosphatase-1G gene locus has been associated with alcohol use disorder (Ruggeri et al., 2015). But whether these modifications are specific to blood cells or to a particular drug is still not clear.

Epigenetic regulations may explain the maintenance of long-term adaptation and a better understanding of the mechanisms across drugs of abuse will help highlight targets for relapse treatment. Manipulation of histone acetylation levels using pharmacological inhibitors of HDACs was assessed in preclinical cocaine studies. Such HDAC inhibitors reduced cocaine intake and motivation in self-administering rats while no effect of the inhibitor was observed on sucrose intake (Romieu et al., 2008). Reduced reinstatement of cocaine-seeking behavior was also observed following 3 weeks of abstinence (Romieu et al., 2011). Extinction of cocaine-induced CPP was accelerated following a similar treatment (Malvaez et al., 2010).

In contrast, opposite results were obtained in other studies on cocaine intake (Sun et al., 2008) and cocaine-induced hyperlocomotion (Kumar et al., 2005). These results point to the importance of the specificity of these inhibitors or the type of treatment used, that may explain distinct effects on addictive responses. Therefore, the need for selective inhibitors of HDAC and controlled paradigms seem crucial to specifically target subtypes of HDAC in therapeutic approaches. Also, differential regulations were observed, depending on the targeted brain structure or the type of drug, as illustrated for HDAC5 with distinct regulations following methamphetamine in DS compared to cocaine in NAc (see above (Li et al., 2017). These observations reveal the complexity of such therapeutic strategies. Another approach could be to target histone acetyltransferase. These enzymes, like CBP, play a role in cocaine action in the NAc (Malvaez et al., 2011) but no study has evaluated their potential efficacy in drug-induced responses. Activation of CBP has recently been shown to reverse deficits in memory processes and promote neurogenesis (Chatterjee et al., 2013). Effects of activation of histone acetyltransferase on drug-induced phenotypes could be proposed as a therapeutic approach.

Several tools are currently available to edit epigenetic factors, like ZFP, TALENs and CRISPR (reviewed in (Waryah et al., 2018). Such approaches provide accurate modification of a given type of epigenetic marks at a precise gene locus, allowing subtle changes to be detected. In vivo, such tools are able to reverse memory deficits in a mouse model of Alzheimer's disease (Bustos et al., 2017). In addictive disorders, one recent study by Nestler's group examined the role of cocaine-induced histone modifications at the gene locus for cyclin-dependent kinase 5 *cdk5*, a critical gene in reward related behaviors (Heller et al., 2016). The authors used zinc finger proteins-genome editing targeting H3K9/14ac, an active mark or H3K9me2, a repressive mark, in vivo and demonstrated a causal role of *Cdk5* epigenetic remodeling in NAc in *Cdk5* gene expression as well as in reward and stress behaviors. Their results revealed novel indicators of a role of this gene in addictive behaviors and, highlighted that targeted epigenetic remodeling approaches represent useful tools for studying molecular changes involved in addictive responses. Neuroepigenetic editing thus represents an emerging tool that will be developed to further investigate cell-type and gene specific adaptations.

7-Concluding remarks

In summary, genome-wide studies have highlighted the implication of several types of neuroepigenetic modifications associated with drugs of abuse, which may have an impact on addictive behaviors. Histone acetylation and methylation are clearly altered by psychostimulants. DNAm and hydroxymethylation represent dynamic mechanisms that may affect enhancers, promoters, splice-sites with different outcome on gene expression. Further, analysis of 3D structure of chromatin brings new insight into the mechanism by which drugs alter gene expression. Combining chromatin architecture analyses with epigenetic and transcriptomic analyses is crucial for a better understanding of these adaptations.

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Figure legends

Figure 1. A) Histone proteins (H2A, H2B, H3 and H4) form an octamer with two copies of each, which is wrapped around 147bp of DNA to form a functional unit of chromatin, the nucleosome. Modifications such as methylation (Me), bimethylation (2Me) , trimethylation (3Me) or acetylation (Ac) can alter the N-terminal tails of these histones (here, H3 is depicted). These modifications lead to either an open (enable gene expression) or close chromatin state (repress gene expression). B) Addition of a methyl group to a cytosine (Me, position 5) can alter the DNA segment activity. When a promoter is highly methylated, this modification often leads to decrease gene expression. The methyl groups can be oxidized by the ten-eleven translocation enzymes family to form a hydroxymethyl group (hMe).

Figure 2. Representative scheme illustrating some critical factors for the analysis of neuroepigenetic modifications by drugs of abuse (cocaine, MDMA, alcohol, cannabinoids and opiates). Species, sex and age of the individuals can modify observed effects. Several paradigms of drug administration are classically used, with distinct timing (acute, chronic, abstinent) to study direct drug effects or relapse, various modes of administration (oral, vapor, intraperitoneal or intravenous), with passive or voluntary access to the drugs (choice or operant system). They all enable to study addiction-related behavioral responses. The genome-wide analysis can be performed on whole brain or microdissected samples to focus on specific brain structures. Recent technologies allow now to lower cellular heterogeneity and focus on specific cell types. A: acute, C: chronic, W: withdrawal.

BOX

Transcriptomic profiling (see (Hitzemann et al., 2013; Wang et al., 2009)

RT-qPCR, gene expression analysis for candidate genes

RNA microarrays, initial genome-wide gene expression profiling using oligonucleotide arrays and hybridization-based approaches.

RNA-Seq, a high throughput sequence-based method. RNA are converted into libraries of cDNA and each molecule is further sequenced with read between 30 to 400 bp. Reads are then aligned to a reference genome. It allows to analyze alternative splicing , the expression of coding and non-coding RNAs with a higher base-pair resolution

DNA methylation profiling see (Kurdyukov and Bullock, 2016)

Whole-genome methylation, approaches to quantify the amount of deoxycytidine (dC) and methylated cytosines (5mC) in a hydrolysed DNA sample using high performance liquid chromatography (ultraviolet), liquid chromatography coupled with mass spectrometry, pyrosequencing LINE-1. These methods do no offer a precise quantification of methylation levels.

Bisulfite sequencing, conversion by bisulfite of cytosine into uracil while 5-methylcytosines are not affected. Following conversion, either whole genome bisulfite sequencing or reduced representation bisulfite sequencing where CpG regions are enriched can be processed.

Methylcytosine binding proteins (MBD) and antibodies targeting 5mC (MeDIP), approaches allowing enrichment of differentially methylated regions (DMR), can be performed before bisulfite treatment.

DNAm microarrays, following bisulfite conversion, allows using specific microarrays to interrogate region-methylation levels such as promoters or gene bodies.

DNA digestion, particular endonucleases are able to digest CCGG sequence to analyze either whole-genome or specific gene methylation levels.

Chromatin profiling see (Park, 2009)

ChIp, Chromatin immunoprecipitation, state of the art technique to study chromatin alterations such as DNA-binding proteins, histone modifications and nucleosomes alterations.

qChIP, investigats a single locus by qPCR following chromatin immunoprecipitation.

ChIP-ChIP, interrogates selected-region modifications using DNA microarrays.

ChIP-Seq, whole-genome sequencing following chromatin precipitation with high base-pair resolution.

DamID-Seq, indentification of DNA adenine methyltransferase without immunoprecipitation step, based on a fusion protein and E. coli DNA adenine methyltransferase; limited to *in vitro* models.

ATAC-Seq / DNA-Seq, addresses chromatin accessibility, providing similar information to RNA-seq but focusing on DNA; used for profiling enhancer regions.

Chromosome conformation capture (see (Davies et al., 2017; Dekker et al., 2017)

3C, analysis of interactions between a single pair of genomic loci.

4C, identification of interactions between one locus and all other genomic loci.

5C, study of functional contacts between all the genes within a given region.

Hi-C, identification of functional contacts through all parts of the genome.

Imaging techniques, mapping of chromatin interactions.

Table 1: Whole-genome epigenetic studies following psychostimulant exposure?

		Analysis	Species	Administration paradigm	Dose	Region	Reference
Cocaine		DNAm microarray	Rat	C, 10d i.v W, 1,30d	0.75mg/kg	NAc	*(Massart et al., 2015)*
		DNAm microarray	Mouse	A, i.p C, 8d C 7d, W 7d, A	20mg/kg	NAc	*(Maze et al., 2010)*
		MBD-seq	Mouse	C, 12d i.v W, 1,21d	0.5mg/kg	mPFC	*(Baker-Andresen et al., 2015)*
		MBD-seq	Rat	C, 13d i.v	0.33mg/kg	mPFC	*(Fonteneau et al., 2017)*
		5hmc-seq RNA-seq ChIp-seq	Rat	C, 7d i.p	20mg/kg	NAc	*(Feng et al., 2015)*
		RNA microarray	Human			Midbrain	*(Bannon et al., 2015)*
		RNA microarray	Mouse	C, 7d e.o.d i.p	20mg/kg	NAc	*(Bu et al., 2012)*
		RNA microarray	Rat	C, 10d i.v W, 1,10,100d	1.5mg/kg	NAc, mPFC	*(Freeman et al., 2010)*
		RNA-seq	Mouse	C, 7d i.p	20mg/kg	NAc, Striatal PSD	*(Eipper-Mains et al., 2011)*
		RNA-seq	Mouse	C, 7d i.p W, 28d	20mg/kg	NAc	*(Eipper-Mains et al., 2013)*
		RNA-seq	Mouse	C, 8d e.o.d i.p E, 8d W, 8d R, i.p	5mg/kg 1.25mg/kg	NAc	*(Lo Iacono et al., 2016)*
		RNA-seq	Mouse	C, 7d i.p	20mg/kg	NAc	*(Wang et al., 2016b)*
		RNA-seq	Mouse	C, 7d i.p	20mg/kg	NAc	*(Cates et al., 2017)*
		RNA-seq	Rat	C, 14d	0.5mg/kg	NAc	*(Zhang et al., 2016)*
		ChIp- ChIp	Mouse	C, 7d i.p	20mg/kg	NAc	*(Renthal et al., 2009)*
		ChIp-seq	Human			Hipp	*(Zhou et al., 2011)*
		ChIp-seq RNA-seq	Human			Hipp	*(Farris et al., 2015)*
		ChIp-seq	Mouse	C, 7d i.p	20mg/kg	NAc	*(Maze et al., 2011)*
		ChIp-seq	Mouse	C, 7d i.p	20mg/kg	NAc	*(Ferguson et al., 2015)*
		ChIp-seq	Mouse	C, 7d i.p	20mg/kg	NAc	*(Damez-Werno et al., 2016)*
		ChIp-seq RNA-seq	Mouse	C, 7d i.p	20mg/kg	NAc	*(Feng et al., 2014; Hu et al., 2017))*
		ChIp-seq	Mouse	C, 7d i.p	20mg/kg	NAc	*(Sun et al., 2017)*
		4C-seq	Rat	C, 7d i.p	20mg/kg	NAc	*(Engmann et al., 2017)*
Methamphetamine	*	DNAm microarray	Mouse	C, 27d e.o.d i.p	0.5 to 4mg/kg	Hipp	*(Itzhak et al., 2015)*
		5hmc-seq	Rat	C, 20d e.o.d i.v	0.1mg/kg	NAc	*(Cadet et al., 2017)*
		RNA microarray	Rat	A, i.p	20mg/kg	NAc	*(Martin et al., 2012)*
		RNA-seq	Rat	C, 14d i.p	1mg/kg	PFC, OFC,	*(Mychasiuk et*

			W, 14d		NAc	*al., 2013)*
	RNA-seq	Mouse	C, 5d i.p W, 2d	2mg/kg challenge: 5mg/kg	NAc	*(Zhu et al., 2015)*
	ChIp-seq RNA microarray	Rat	A, i.p	2x5mg/kg	Striatum	*(Cadet et al., 2013)*
MDMA	RNA microarray	Mouse	C, 11d i.v	0.125mg/kg	NAc, FC, dorsal raphe nucleus, Hipp	*(Fernandez-Castillo et al., 2012)*

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year)

Table 2: Whole-genome epigenetic studies following alcohol exposure

		Analysis	Species	Administration paradigm	Dose	Region	Reference
Alcohol		DNAm microarray	Human			FC	*(Manzardo et al., 2012)*
		DNAm microarray	Human			Precuneus	*(Hagerty et al., 2016)*
		DNAm microarray	Human			PFC	*(Wang et al., 2016a)*
		Bis-seq	Rhesus macaques	C, 1y oral	4%	NAc	*(Cervera-Juanes et al., 2017a, b)*
	*	Bis-seq	Rat	2cycles of C, 3d + 3d oral W, 2d	20%	Hypo	*(Asimes et al., 2017)*
		RNA microarray	Human			PFC	*(Zhang et al., 2014)*
		RNA-seq	Human			Hipp	*(Enoch et al., 2013; Enoch et al., 2014)*
		RNA-seq	Mouse	C, 60d e.o.d oral	15%	Microglial cells	*(McCarthy et al., 2018)*
		RNA-seq	Rat	C, 56d oral C, 28d oral + 21d gavage	6% 6% + 30%	NAc	*(Morud et al., 2017)*
		RNA-seq	Rhesus macaques	C, 1y oral	4%	CeA	*(Iancu et al., 2018)*
		RNA-seq	Mouse	4 cycles of C, 4d inhalation W, 2d C, 5d oral	inhalation 16h/d 15%	NAc, Striatum, BNST, Amy, VTA, PVC	*(Mulligan et al., 2017)*
	*	RNA-seq RNA microarray	Rat	A: 2inj at GD12 i.p	2.9g/kg + 1.45g/kg	Amy, NAc	*(Ignacio et al., 2014)*
		RNA microarray	Rat	PD4 to PD9 2inj/d oral	2.625 g/kg	Hipp	*(Balaraman et al., 2017)*
		RNA microarray	Mouse	A, i.p	1.8-g/kg	Hipp	*(Baker et al., 2017)*
		RNA microarray	Mouse	A, PD4&7 2inj/d s.c	2x2.5g/kg	Hipp	*(Chater-Diehl et al., 2016)*
	*	RNA microarray	Mouse	C, 8d oral	10%	Hipp	*(Marjonen et al., 2015)*
	*	RNA microarray	Rat	A, 2inj at GD12 i.p	2.9g/kg + 1.45g/kg	Amy, NAc	*(Middleton et al., 2012)*
		RNA-seq	Mouse	*Selective breeding of mice for ethanol preference*		NAc	*(Colville et al., 2017)*
		RNA-seq	Rat	*Predisposition for ethanol preference*		Whole brain	*(Saba et al., 2015)*

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year; PD: postnatal day; GD: gestational day; CeA: central amygdala)

Table 3: Whole-genome epigenetic studies following opiate exposure

		Analysis	Species	Administration paradigm	Dose	Region	Reference
Morphine		RNA microarray	Mouse	A, i.p C, 5d ; 3/d i.p	20mg/kg 10,20,40mg/kg	Striatum	*(Korostynski et al., 2007)*
		RNA microarray	Mouse	C, 5d s.c	25mg pellet	NAc	*(Grice et al., 2007)*
		RNA microarray	Mouse	C, i.p 6d	20-100mg/kg	Extended amygdala	*(Befort et al., 2008)*
		RNA microarray	Mouse	S, 6h s.c C, 4d s.c	25mg pellet	Hypo	*(Anghel et al., 2010)*
	*	RNA-seq	Rat	C, 21d i.v E, 10d R, i.p	0.25,0.75,1.25mg/kg 1 mg/kg	NAc	*(Vassoler et al., 2017)*
Heroine	*	DNAm microarray	Human			DLPFC	*(Chatterton et al., 2017)*
		DNAm microarray	Human			OFC	*(Kozlenkov et al., 2017)*
		RNA microarray	Human			NAc	*(Egervari et al., 2017)*
		RNA microarray	Rat	C, 7d i.p	1mg/kg	NAc	*(Yan et al., 2017)*
		RNA-seq	Rat	C, 19d i.v E,1d	0.06mg/kg-19d	NAc	*(Imperio et al., 2016)*

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year;)

Table 4: Whole-genome epigenetic studies following cannabinoid or nicotine exposure

		Analysis	Species	Administration paradigm	Dose	Region	Reference
THC	*	Bis-seq	Rat	C, PD28 to PD49 e.o.3d i.p	1.5mg/kg	NAc	*(Watson et al., 2015)*
		RNA microarray	Mouse	C, 4.5d i.p	10mg/kg	Cerebellum	*(Colombo et al., 2009)*
Nicotine	*	RNA microarray	Mouse	C, 21d oral W, 90d	200µg/ml	Cerebral cortex	*(Jung et al., 2016)*
		RNA-seq	Human			DLFC	*(Tao et al., 2017)*
		RNA-seq	Mouse	C, 14d s.c	2mg/kg/h mini osmotic pumps	SNc neurons	*(Henley et al., 2013)*
	*	RNA-seq	Mouse	C, 5d by inhalation	13-16mg/ml	FC	*(Lauterstein et al., 2016)*
	*	RNA-seq	Mouse	C, 28d oral	200µg/ml	*Pomc* neurons	*(Silva et al., 2016)*
		RNA-seq	Rat	C, 14d i.p	0.3mg/kg	PFC, OFC, NAc	*(Mychasiuk et al., 2013)*

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year; PD: postnatal day)

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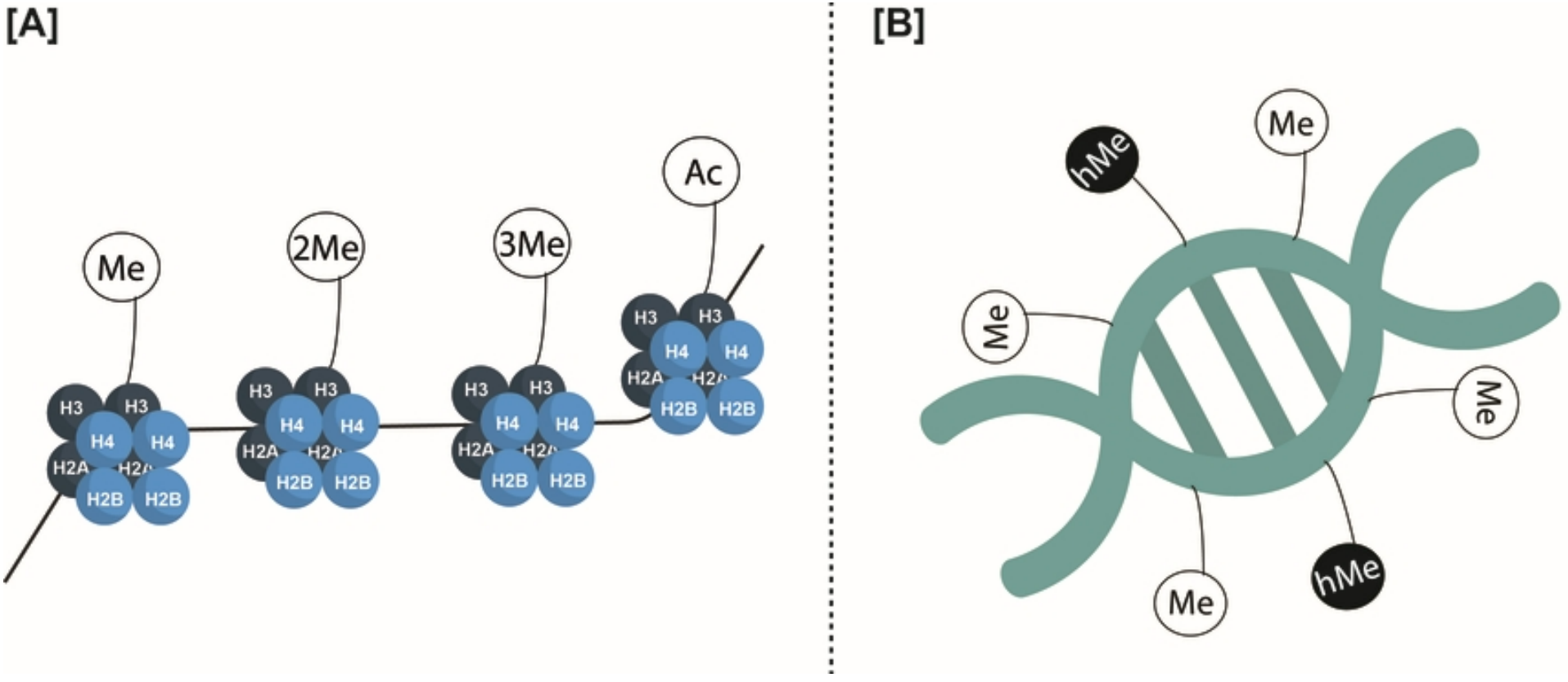
[A]
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hMe

Drugs

Cocaine

MDMA

Alcohol

Cannabinoids

Opiates

Species

Mouse

Rat

Non-human primate

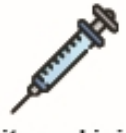
Human

Paradigm of administration

Timing

Oral
(A, C, W and Operant)

Vapor chamber
(C and W)



Intraperitoneal injections
(A, C and W)

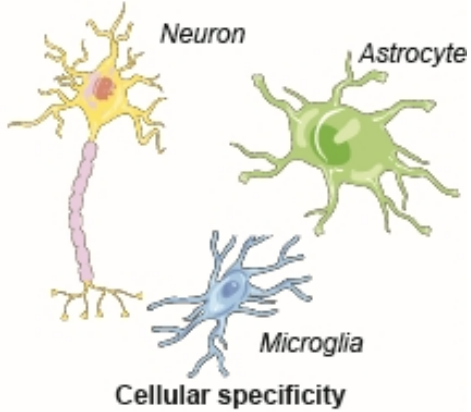
Intravenous injections
(C, W and Operant)

Target

Cell diversity

Whole brain sample
(High cell heterogeneity)

Brain structure
(Intermediate cell heterogeneity)



Cellular specificity