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Epigenetic Mechanisms of Drug Addiction

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The Epigenetic Mechanisms of Amphetamine

Abstract

Amphetamine (AMPH) is a psychostimulant and the most prescribed drug to treat attention deficit hyperactive disorder (ADHD). Although therapeutically used doses are generally well tolerated, numerous side effects are still known to occur, such as jitteriness, loss of appetite and psychosis. Moreover, AMPH is liable to be abused by users looking for increased alertness, weight loss or athletic performance. A growing body of evidence indicates that drugs of abuse, including AMPH, control gene expression through chromatin modifications. However, while numerous studies have investigated the molecular mechanisms of AMPH action, only a small number of studies have explored changes in gene expression caused by AMPH. This review examines the epigenetic changes induced by chronic and acute treatments with AMPH and includes, where relevant, data obtained with other psychostimulants such as methamphetamine and cocaine.

Introduction

Though an addictive compound, amphetamine (AMPH) is a drug that has been used to treat a variety of diseases such as narcolepsy, obesity and ADHD [1,2]. Initially described as a potent sympathomimetic drug with cardiovascular and gastrointestinal effects, it soon became clear that AMPH has reinforcing properties leading to widespread abuse [3]. AMPH readily crosses the blood brain barrier and stimulates the mesolimbic and mesocortical pathways (reward system) where it raises the synaptic concentration of the neurotransmitters dopamine (DA), norepinephrine (NE) and to a lesser extent serotonin (5-HT) [4,5]. Under normal conditions, the extracellular concentration of these neurotransmitters is maintained at low levels by their respective neurotransmitter transporters, namely DAT, NET, and SERT, which rapidly reuptake the neurotransmitters inside the cells [6,7]. AMPH is a substrate for all three neurotransmitter transporters; therefore, via competitive inhibition, it prevents the reuptake of DA, NE and 5-HT. Although AMPH can act on DAT, NET and SERT, it is well established that its stimulant effects are primarily mediated by its ability to bind and reverse the release of DA through the DAT [8,9]. In fact, a common feature among all drugs of abuse is their ability to increase the extracellular concentrations of DA in the reward system, and this phenomenon is believed to be the first step in generating addiction [4].

Genome-wide analyses such as microarrays and RNA-sequencing following acute and chronic exposure to drugs of abuse have demonstrated striking changes in expression of genes that affect key regions of the brain involved in reward [10-15]. These studies have suggested that AMPH’s ability to alter gene expression is an important step in the initiation, maintenance and relapse to addictive behaviors. For instance, several studies have shown that drugs of abuse induce a transient increase of the FOS family transcription factors, including c-fos, FosB and the truncated splice variant ΔFosB in rats and mice striatum [16-18]. All members of the FOS family form heterodimers with proteins of the JUN family, resulting in the formation of the Activator Protein-1 (AP-1). Following binding to cognate DNA sites in the promoter regions of genes, AP-1 can either activate or repress genes.

Recent studies have shown that a single dose of AMPH increases c-fos expression in the murine striatum [19-21]. Interestingly though, chronic (7 days) AMPH treatments causes instead a strong decrease of c-fos expression [19,20], and a several fold increase of ΔFosB [19,22], suggesting that acute and chronic AMPH exposures regulate the expression of c-fos through different and possibly opposite mechanisms. The higher expression of ΔFosB after continuous drug exposure and its persistence after several days of withdrawal has suggested that ΔFosB might function as a general molecular switch in the development of addiction [23-25]. In fact, ΔFosB signal has been shown to persist also after several days of cocaine withdrawal, and eventually disappear after one to two months. Because of the importance of transcription factors in regulating gene expression and the access to more affordable genome sequencing technologies, an increasing number of epigenetic studies have emerged in the field of drug addiction in the last decade. In fact, unraveling the mechanisms through which drugs of abuse manipulate gene expression will help us to understand how drugs of abuse induce addiction.

Epigenetic Mechanisms

The term epigenetics, as it is currently used, refers to regulatory mechanisms that contribute to or are associated with changes in gene expression that do not involve alterations to the DNA sequence. These mechanisms, being “above the gene”, include changes to chromatin structure, i.e. histone modifications and nucleosome positioning, DNA methylation and hydroxymethylation, and non-coding RNA-dependent mechanisms. Collectively, the sum total of all the epigenetic changes in the genome of a cell is referred to as the ‘epigenome’. Several groups in recent years have begun to investigate how changes to the epigenome contribute to the mechanisms of action through which drugs of abuse generate addiction. Indeed, there is mounting evidence suggesting that changes in the cellular environment caused by drugs result in activation or repression
of genes. These changes in gene expression are then maintained even in the absence of the stimulating signal. For instance, a recent study demonstrated that when male rats voluntarily ingested cocaine, their male, but not female, offspring exhibited increased levels in protein and mRNA of the brain-derived neurotrophic factor (Bdnf) in the medial prefrontal cortex [26]. Moreover, Feng et al. showed that mice chronically treated with cocaine exhibited a unique chromatin signature which correlated with altered gene expression. Interestingly, they found that cocaine treatments altered expression of only about 100 genes, but it induced alternative splicing in over 1700 genes [27]. Collectively, these findings highlight the importance of understanding the epigenomic changes induced by drug exposures.

In this review, we will summarize research findings that describe how the three major mechanisms of epigenetic regulation, chromatin structure and histone modifications, DNA methylation, and non-coding RNAs, have been linked to gene expression changes following AMPH use (Table 1).

### Chromatin Structure

In eukaryotes, the negatively charged genomic DNA associates with a group of small, positively charged proteins named histones (H). The result of this interaction is a nucleosome, 147 base pairs of DNA wrapped around a protein octamer comprised of two sets of H2A, H2B, H3, and H4 (Figure 1) [28]. Nucleosomes and DNA are condensed in a highly complex structure, known as chromatin, which forms chromatin fibers of about 10 nm in diameter. The chromatin fibers can then be further condensed by coiling into 30 nm fibers. The extent of chromatin condensation is highly dynamic and varies during the life cycle of the cell, e.g. during important cellular functions such as transcription and replication. Cells modulate gene expression via protein complexes that can modify the chromatin in a non-heritable fashion. These chromatin remodeling complexes are often recruited to their target genes by DNA-binding transcription factors. The chromatin remodeling complexes can alter chromatin structure in many ways: (a) they can modify specific residues of the N-terminal tails of the histone core proteins by acetylation, methylation, phosphorylation, ubiquitination, etc. thus altering their affinity for DNA; (b) they can introduce variant histones into the chromatin, thus contributing a unique or distinct nucleosomal architecture, which in turn can activate or repress gene expression; (c) they can utilize the energy of ATP hydrolysis to either allow or limit access to DNA sequences; or (d) in order to completely silence gene activity, additional mechanisms such as DNA methylation have evolved, which results in the establishment of a highly quiescent state at genes. Often, these different mechanisms occur in combination. For instance, epigenetic marks such as methylation of the histone H3

<table>
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<td></td>
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<td>Increased mir-181a expression, which modulated the expression of the glutamate receptor 2 subunit</td>
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HDAC: Histone Deacetylase; HDACi: HDAC inhibitor; PFC: Prefrontal Cortex; OFC: Orbital Frontal Cortex; NA: Nucleus Accumbens; CP: Caudate Putamen; miR: microRNAs
lysine 9 residue (H3K9 trimethylation) and DNA methylation are associated with repressed and/or silenced genes [29]. Moreover, synergetic connections have also been established between histone H3K9 trimethylation at lysine 9 and DNA methylation in many model systems [30].

**Histone Modifications**

Recent studies have demonstrated that drugs of abuse induce chromatin rearrangements. For instance, acetylation has been the most reported change at histones during exposure to the psychostimulant AMPH (Table 1). Acute AMPH treatments were shown to increase H4 acetylation at the c-fos promoter [19], and this increase disappears after chronic AMPH treatments. As histone deacetylation has been related to gene silencing, these results suggest histone deacetylation mediates the repression of c-fos expression following chronic AMPH treatments. This conclusion was supported by the fact that histone deacetylase (HDAC) inhibitors reversed the AMPH-induced reduction of c-fos expression observed after chronic treatments [19]. Taken together, these data suggest that in drug-naive animals AMPH increases gene expression of c-fos by increasing acetylation of the H4 at the c-fos promoter whereas, prolonged treatments with AMPH promote gene silencing by inhibiting acetylation.

Studies reporting histone acetylation have been particularly prolific thanks to the use of HDAC inhibitors such as butyric acid (BA) and valproic acid (VPA). Because of their ability to readily cross the blood brain barrier, HDAC inhibitors have been broadly used both in in vivo and in vitro studies to investigate whether and how histone acetylation changes psychostimulant-induced behaviors (Table 1). In 2007 Kalda et al. demonstrated that while BA or VPA do not generate locomotor effects in mice when administrated alone, they potentiated the locomotor sensitization induced by repeated AMPH treatments [31]. These results correlated with in vitro data showing that co-treatment with VPA and AMPH further increased H4 acetylation with respect to the levels of acetylation obtained when VPA or AMPH were administrated individually. Another study pointed out that AMPH and HDAC inhibitors produce synergetic effects [22]. In this study, the authors showed that mice chronically treated with AMPH exhibited HDAC inhibitor-like effects, i.e. increased global acetylation on lysine 12 at histone 4 (H4K12ac) in the striatum and specific H4K12ac at the fosB promoter. On the other hand, chronic treatments with HDAC inhibitors caused AMPH-like effects, i.e. increased protein levels of phosphorylated cAMP responsive element binding protein (CREB), and increased ΔFosB proteins in the striatum [22]. Moreover, when AMPH and HDAC inhibitors were administered together, the levels of H4 acetylation and phosphorylated CREB were further increased. As CREB and ΔFosB are molecular markers of neuroadaptation caused by chronic use of psychostimulants [32], the authors investigated a potential common mechanism underlying the similar effects seen after HDAC inhibitors and AMPH treatments. Immuno-precipitation experiments demonstrated that repeated AMPH or VPA treatments decreased the amount of CREB proteins interacting with a member of HDAC family, HDAC1. When AMPH was administered together with VPA, the number of CREB proteins bound to HDAC1 decreased even further. These data suggest that both AMPH and HDAC inhibitors alter CREB phosphorylation, which in turn alter the interaction between HDAC1 and CREB. The authors also investigated the effects that HDAC inhibitors had on AMPH-induced hyper-locomotion. In accordance with data published previously [31], Shen et al. found that co-treatment with AMPH and HDAC inhibitors prolonged
DNA methylation has been only recently attributed to the gene \[\text{H3K9me2}\] at the c-fos promoter. Interestingly, under the same conditions the c-fos promoter also exhibited reduced levels of acetylation at the histone 4, which resulted in a reduction of c-fos mRNA expression [19]. These effects were probably mediated by the enzyme HDAC1 as chronic AMPH treatments caused an increase of HDAC1. Taken together, these results demonstrate that following chronic AMPH treatment, various repressive modifications work co-operatively to decrease c-fos mRNA [19].

The results reported above are the only example, as to date, of histone methylation induced by AMPH. However, histone methylation changes have been broadly described following treatments with various psychostimulants [27,43]. For instance, a significant increase of methylation at lysine 4 of histone 3 (H3K4me3) was reported in protein extracts of the dorsal striatum and at the promoter for the chemokine receptor CCR2 gene following methamphetamine treatments [44]. This increase of H3K4me3 correlated with an increase of CCR2 mRNA [43]. However, chromatin immunoprecipitation (ChIP) assays did not show significant changes in H3K4me3 at the promoters of c-fos, fosb, c-jun, and Bdnf. Further investigation is needed to understand the physiological impact of these AMPH-induced epigenetic modifications.

**DNA Methylation and Hydroxymethylation**

DNA methylation refers to the addition of a methyl group to the cytosine or adenine DNA nucleotides. In adult somatic cells, DNA methylation normally occurs on cytosines in CpG dinucleotides [45]. It has been suggested that DNA methylation may affect the transcription of genes in different ways which in general leads to reduction in gene expression. In fact, the inclusion of a methyl group into the DNA may block the binding of transcriptional proteins to the gene [46], and/or facilitate the binding between the DNA and proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins can in turn recruit other remodeling proteins to the locus, for example histone deacetylases. DNA methylation has been only recently considered as a possible epigenetic mechanism activated by AMPH (Table 1). One study, investigating the persistency of gene expression after drug withdrawal, found that global DNA methylation was increased in rat nucleus accumbens, orbital frontal cortex, and medial prefrontal cortex two weeks after chronic (14 days) AMPH treatment [47]. These results suggest that gene repression might underlie the long-term effects caused by the use/abuse of psychostimulants. Interestingly, a recent study, investigating the epigenotype of offspring exposed to methamphetamine in utero, found that methamphetamine causes DNA hypermethylation in promoters of genes involved in learning, memory and synaptic plasticity [48]. These studies indicate that the methamphetamine-induced long-term effects might be mediated by alterations in DNA methylation.

DNA methyltransferase (Dnmt) enzymes catalyze methylation of the DNA. This family of proteins uses S-adenosyl methionine as donor of the methyl group. In mammals three Dnmt enzymes have been identified and they are named Dnmt1,
Dnmt3A and Dnmt3B. Dnmt2 was recently shown to methylate RNA rather than DNA [49]. There is a fifth protein, named Dnmt3L, closely related to Dnmt3A and Dnmt3B, which is critical for DNA methylation but is not active on its own. As of today, no study has investigated the effect of AMPH on Dnmt activity, however Numachi et al. demonstrated that methamphetamine is capable of changing the expression of Dnmt1 [50]. They showed that acute and chronic methamphetamine treatments changed Dnmt1 levels in rat nucleus caudate, hippocampus, nucleus accumbens, and cerebellum. Considering the functional and structural homology between AMPH and methamphetamine, these results suggest that AMPH might change Dnmt1 activity as well. Notably, in the same study, the authors also reported that methamphetamine caused different expression patterns of Dnmt1 in the two strains of rats they tested, Fisher 344/N and Lewis/N. Specifically, acute and chronic methamphetamine increased the mRNA of Dnmt1 in the nucleus caudate and nucleus accumbens of Fisher 344/N rat, whereas an increase in the cerebellum was observed only after acute treatments. In the same animals, methamphetamine caused a decrease in Dnmt1 mRNA in the hippocampus. On the other hand, the Dnmt1 mRNA in Lewis/N rats was decreased in the nucleus caudate following acute and chronic methamphetamine treatments, and no change was observed in the nucleus accumbens and cerebellum. Finally, in the Lewis/N hippocampus an increase in Dnmt1 mRNA was measured only after acute treatments. The authors suggested that the differing patterns of the measured responses might be due to a difference in levels of feedback in the hypothalamic-pituitary-adrenocortical axis between the two strains. The observation that the methamphetamine-induced changes in Dnmt1 vary across different strains of rat unravels a higher level of complexity of the mechanisms through which psychostimulants cause epigenetic modifications.

Methyl CpG-binding protein-2 (MeCP2), a protein repressing transcription activity, is another protein recruited after methylation of DNA occurs. The importance of MeCP2 in normal brain development is highlighted by the fact that in humans, mutations in MeCP2 cause the autism spectrum disorder and Rett syndrome [51]. MeCP2 has been previously shown to be essential in learning and memory formation, and is now being explored as a modulator of addiction [52]. Deng et al. investigated the effect of AMPH on MeCP2 in adult mice, and found that AMPH induces a transient increase of phosphorylated MeCP2 in a small population of neurons distributed through the nucleus accumbens of mice [20]. If mice were pre-treated with the D1 receptor antagonist SCH22390 the AMPH-induced increase of phosphorylated MeCP2 was partly reduced to control levels, suggesting that activation of the D1 receptors mediates MeCP2 phosphorylation. Also, the authors showed that viral manipulation of MeCP2 expression in the nucleus accumbens modulates AMPH-induced conditioned place preference (CPP). Specifically, lentiviral overexpression of MeCP2 inhibited the AMPH-induced CPP, whereas lentiviral knockdown of MeCP2 augmented both AMPH-induced CPP and locomotor activity. Furthermore, mice bearing a hypomorphic mutation in MeCP2 exhibited altered behavioral response to both acute and chronic AMPH treatments and obstructed dendritic plasticity induced by repeated AMPH treatments [20]. These results demonstrate that MeCP2 is a crucial component in the behavioral response to AMPH.

Recently, 5′-hydroxymethylation of cytosine (5-hmC, the ‘sixth base’) was revealed to be a new player in the epigenetic field [53]. The role of 5-hmC is of particular interest in mental disorders because it has been shown to be specifically enriched in brain tissues [54]. Further, 5-hmC is inversely correlated with MeCP2 expression [55,56]. There is no information regarding the role of 5-hmC in gene expression changes following AMPH exposure. However, given that AMPH treatment in hypomorphic Mecp2 mutant mice resulted in reduced locomotor sensitization and place conditioning, and the inverse relationship of MeCP2 and 5-hmC, it is reasonable to hypothesize that there might be a significant association between AMPH treatment and 5-hmC.

Non-coding RNAs

The discovery of RNA interference phenomenon by Craig Mello, Andy Fire and their colleagues [57] highlighted two main observations: 1) both sense and antisense RNA preparations can cause interference and reduce gene expression, and 2) RNA interference persisted well into the next generation [57]. Since their discovery, the field has exploded with the discovery of a number of non-coding RNAs, including micro-RNAs (miRNAs), long, non-coding RNAs (lncRNAs), piwi-RNAs (piRNAs), and more recently, circular RNAs (circRNAs) [58], which serve as miRNA ‘sponges’ [59]. Originally discovered in *C. elegans* [60], the miRNA were then proven to exist in mammals as well. miRNAs are small RNAs which bind to complementary sequences on their target mRNAs, thus repressing translation and silencing gene expression. miRNAs have been also implicated in the mechanism of action of drugs of abuse, and changes in miRNAs following drug exposure has been shown to down regulate the expression of genes that are thought to be involved in drug addiction [61-63]. As of today, two studies have reported miRNA changes following AMPH treatments (Table 1). Saba et al demonstrated that in mice treated for five days with AMPH or cocaine the expression of miRNA increased in a drug-dependent and brain region-specific manner [64]. Specifically, they showed that AMPH treatment increased the expression levels of a number of miRNAs and, particularly miRNA 181a (miR-181a), in four areas of the brain receiving dopaminergic inputs from the ventral tegmental area: ventral mid-brain, subcortical limbic forebrain, prefrontal cortex and the hippocampus. As miR-181a is also induced by cocaine in the dopamine D2-like receptors expressing neurons [65], these results suggest that miRNA might have a role in drug addiction. Moreover, Lippi et al. showed a robust and significant increase of miR-29b, miR-142/5p, miR-183, miR-198, miR-215, miR-216, miR-217 and 292/3p in the hippocampus of mice treated with AMPH for five days [66]. Using the same experimental paradigm, the authors also reported that miR-142/5p and miR-216b were strongly increased after cocaine or nicotine treatments, suggesting that these two miRNAs are common markers of addictive drugs.

Conclusions

Epigenetic modifications caused by addictive drugs play an important role in neuronal plasticity and in drug-induced
behavioral responses. Although few studies have investigated the effects of AMPH on gene regulation (Table 1), current data suggest that AMPH acts at multiple levels to alter histone/DNA interaction and to recruit transcription factors which ultimately cause repression of some genes and activation of other genes. Importantly, some studies have also correlated the epigenetic regulation induced by AMPH with the behavioral outcomes caused by this drug, suggesting therefore that epigenetics remodeling underlies the behavioral changes induced by AMPH. If this proves to be true, the use of specific drugs that inhibit histone acetylation, methylation or DNA methylation might be an important therapeutic alternative to prevent and/or reverse AMPH addiction and mitigate the side effects generated by AMPH when used to treat ADHD.

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