Genomics and epigenomics of addiction

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Abstract

Recent progress in the genomics and epigenomics of addiction has contributed to improving our understanding of this complex mental disorder's etiology, filling the gap between genes, environment, and behavior. We review the behavioral genetic studies reporting gene and environment interactions that explain the polygenetic contribution to the resilience and vulnerability to develop addiction. We discuss the evidence of polymorphic candidate genes that confer susceptibility to develop addiction as well as the studies of specific epigenetic marks that contribute to vulnerability and resilience to addictive-like behavior. A particular emphasis has been devoted to the miRNA changes that are considered potential biomarkers. The increasing knowledge about the technology required to alter miRNA expression may provide promising novel therapeutic tools. Finally, we give future directions for the field's progress in disentangling the connection between genes, environment, and behavior.

Keywords

Multifactorial, polygenetic, epigenetic, genes-environment interaction, endophenotype, DNA methylation

1. Introduction

Significant findings from the behavioral genetics field have contributed to our understanding of individual differences in behavior. Research in humans in this area applies studies of monozygotic identical twins, family investigations, and adoption studies. These studies have pointed out that psychiatric disorders, personality traits, and cognition have a significant polygenetic influence. However, the environment and the interaction between both factors are essential. Molecular genetic studies in humans and animals have shown that the susceptibility for psychiatric disorders such as addiction is heritable, highly polygenic and that the combination of environmental and genetic factors contributes to the observed phenotypic variation (Plomin and Daniels, 2011). Thus, addiction has a complex multifactorial pattern of inheritance. This multifactorial genetic model aims to explain how the interaction between multiple gene networks and environmental factors strongly impacts brain function early during development and later throughout the adulthood period influencing behavior (Figure 1). Hence, genes are not single direct triggers of mental disorders but contribute to confer risk for pathological behavior development, accounting for a significant fraction of total variation (Hamer, 2002). In this context, the present paper aims to discuss why some individuals are vulnerable to develop addiction, while others are resilient to certain environmental risk factors, even if access to the drug is present. Several genetic and neurobiological factors of vulnerability and resilience have been identified in human and animal studies. For example, to manipulate a vulnerable phenotype to develop food addiction was generated by manipulating the activity of "top-down" cortical circuits

using chemogenetic approaches, whereas a cell-type gene deletion has promoted resilience to food addiction (Domingo-Rodriguez et al., 2020).

The interaction between genes and environment is now better understood with the recent advances in the epigenetics field. Epigenetics refers to changes that regulate gene expression without causing variation in the DNA sequence. The main epigenetic mechanisms comprise DNA methylation, histone modifications, and non-coding microRNAs (miRNAs). These changes are reversible, but early-life epigenetic modifications of genes in the brain may lead to behavioral patterns of response that can last for life. Indeed, an individual's experience can modify the chromatin topography within the brain in a region-specific and cell-type-specific manner. The environment can produce different gene expression changes, even in genetically identical individuals, such as the monozygotic twins (Nestler, 2014). A significant contribution to the field was the knowledge that these epigenetic signatures may have a transgenerational behavioral transmission pattern, such as in the case of exploratory, nurturing, and anxiety-related behaviors (Meaney and Szyf, 2005). Consequently, epigenetics concedes an explanation for the gap of the pathway from genotype to phenotype. Addiction is a multifactorial complex disorder in which genes and environment interact with each other. Nowadays, we can study genes in sufficient detail to move beyond the nature-nurture dichotomy. Hereditary influences ("nature") comprise variations, polymorphisms in DNA sequences, transmitted from generation to generation over an evolutionary time scale. The environment ("nurture") influences gene expression during an individual's lifetime, which also has a major consequence on the phenotype (Robinson, 2004). Thus, nurture is the personal experience in each individual's life established for the interactions with the environment as childhood, economics, or social relationships, that modifies the genetic

factors defined by nature. In the post-genomic era, there has been a shift within the scientific community from mere acceptance of nature and nurture mutual effects to a more complex understanding of gene–environment interaction. It is well-known that DNA is both inherited and environmentally reactive through different mechanisms, including epigenetics. For behavior, gene expression in the brain is the primary readout of the interaction between genetic and environmental factors. This modulation involves complex probabilistic interactions between genes and the environment, rather than merely additive effects. Understanding that phenotypes are the developmental result of interactions between genes and the environment is essential to avoid oversimplification by focusing on monogenic traits (Haskel-Ittah et al., 2020). Thus, the environment is more salient in influencing the phenotype than predicted and may affect phenotype acting at different levels, including gene regulation via epigenetics and mutations by changing the DNA sequence. This modulation is termed phenotypic plasticity. Classical genetic approaches, such as adoption and twin research-based methods, have identified the influence of the environment controlling genes and the environment separately (Plomin and Daniels, 2011). The influence of a non-shared environment was significant in genetic studies because it made individuals differ although being genetically similar. Indeed, genetic influence never explains all percentages of variance for complex phenotypes such as addiction, and the remaining variance is related to environmental influences. In animal models, the environment's influence has been translationally modeled using childhood separation procedures after birth or stress induction (Meaney and Szyf, 2005). Moreover, the manipulation of genetic factors in animals contributed to an evolving field with a promising future to decipher the neurobiological substrate of complex multifactorial disorders, such as addiction.

Human genetic findings are crucial to discover underlying loci for complex human diseases such as addiction, but these findings are frequently limited to the population under study. Thus, genome-wide association study (GWAS) studies in humans cannot reliably capture complex multifactorial disease neurobiology without further mechanistic studies. These studies must complement animal research to straightforwardly discover molecular networks linked with altered phenotypes with temporal, cell-type-specific, and brain-area resolution. Mice are the leading source for genetic complex disease-related research. Recent progress in DNA-sequencing, DNA-cloning, bioinformatics, viral delivery approaches, and gene editing have advanced the resources of mouse genetics. The link of animal studies with human genetics has been improved in recent years mainly due to the new tools available for molecular discovery and the efforts in translational research. Thus, animal studies are now able to genetic variants discovered in humans to a relevant biological process and often to a pharmacological target. Human and animal genetic studies' connections offer an efficient approach to biological discovery and clinical translation (Saul et al., 2019). The link of animal studies with human genetics has also significantly improved, resulting in new tools for discovering molecular underpinnings and translational research. Thus, animal studies may link a human genetic variant discovered to a relevant biological process and often to a pharmacological target. Human and animal genetic studies' connection offers an efficient and economical approach to biological discovery and clinical translation (Saul et al., 2019).

The current review aims to examine how behavioral genetic research has contributed to advancing our understanding of the links between genes, environment, and behavior and the substrate of resilience and vulnerability to addiction. It is a state-of-the-art review that offers new perspectives on addiction genetics and epigenetics with a comprehensive search of

current literature to underline areas for further research. This review helps to understand the existing state of knowledge and priorities for future research. First, we review the genetics of vulnerability and resilience underlying candidate genes linked to these two distinct dimensions' etiology and developmental profiles. Second, we examine the epigenetics of vulnerability and resilience. At each step, we discuss the strengths and limitations of applying the different methodological approaches, such as GWAS or epigenetics, to investigate individual differences in vulnerability and resilience. Third, we focus on miRNAs as potential biomarkers due to their capacity to be secreted extracellularly and systemic circulation. Finally, we review novel approaches to alter miRNAs expression that, together with the increasing knowledge about miRNAs, may open new promising therapeutic tools.

2. Genetics of vulnerability and resilience in addiction

The genetic factor plays an essential role in the individual susceptibility to develop addiction. The quantitative genetic theory began in the early part of the 19th century to answer the dichotomy between qualitative Mendelian genetics and quantitative normal distributions (Plomin and Daniels, 2011). In the 20th century, this theory progressed, and the liability threshold model emerged, highlighting a normal risk distribution for bimodal effects (affected or non-affected). Later, this model was applied in human genetics and established today's fundamental concepts (Martin et al., 2019). This theory leads to the multifactorial model, where individuals' phenotypes follow a normal distribution, with the majority located in the center of the inverted U-shaped curve and the minorities representing the extremes subpopulations of a resilient or vulnerable continuous phenotypic variation where some individuals may pass a certain threshold and become affected (Figure 2). This model

suggested that several minor genetic and environmental factors, combined interactively, could provide phenotypic variation along a continuum for binary human diseases and traits, introducing the statistical theory of the modern liability threshold model (Martin et al., 2019). Such models are relevant to mostly all common psychiatric disorders, which are not monogenic disorders.

In substance use disorder, classical twin, adoption, and family human studies estimate that 40-60% of the population variability in becoming addicted is attributable to genetic factors (Agrawal et al., 2012). The heritability of cocaine addiction is among the highest among psychiatric disorders, estimated at around 65% for women (Kendler et al., 2000) and 79% for men (Kendler and Prescott, 1998). Concerning other abuse drugs, the heritability calculated was 48-66% for alcohol abuse, 60% for nicotine dependence, and 54% for opioid use disorder (Agrawal et al., 2012; Kendler and Prescott, 1998; Tsuang et al., 1998). The inheritance of addiction does not follow a Mendelian transmission pattern, which means that the heritability is polygenic, involving multiple gene variants, complex networks of gene-gene, epistasis (complex interactions among genetic loci), and gene-environment interactions, among others (Phillips, 2008; Volkow and Muenke, 2012). The advances in the knowledge of the neurobiological substrates underlying substance use disorders have allowed performing hypothesis-driven candidate gene studies to evaluate suspected connections between specific genes and addiction endophenotypes (Caspi and Moffitt, 2006). Endophenotypes are the quantifiable mechanisms alongside the pathway between a complex psychiatric disease and the distal genotype. They can be biochemical, neurophysiological, neuroanatomical, endocrinological, cognitive, or neuropsychological (Gottesman, 2005). Endophenotypes have supposed an advance since they are heritable neurobiological

substrates at an intermediate position in the pathway between the disease and the genotype. They represent more clear traces of genetic mechanisms than the disease syndrome itself, supporting the notion that psychiatric diagnoses can be deconstructed, resulting in a more precise and successful analysis (Gottesman, 2005). Multiple addiction endophenotypes have been studied, but we have focused our review on the reward processing linked to the dopaminergic system, as the most directly related to the initiation of the addictive process taken into account its involvement in the primary rewarding effects of all drugs of abuse and natural rewards. Although all abuse drugs lead to a release of dopamine in the brain reward region, the nucleus accumbens (NAc) is essential to differentiate opioid and psychostimulant use disorders from a behavioral and neurobiological point of view (Badani et al., 2011). One candidate gene widely studied is the *DRD2* gene that encodes for the dopamine-type 2 receptor (D2R), with a specific polymorphism conferring a high risk of 'reward deficiency syndrome,' consisting of a hypodopaminergic state due to reduced levels of D2R density (Blum et al., 2000; Febo et al., 2017). The reduction of D2R levels can produce a decrease of feeling pleasure for usual incentives and a need to compensate for this deficiency, promoting a high risk for multiple addictive, impulsive, and compulsive behaviors overall drugs of abuse (Tsou et al., 2019). Hence, other polymorphisms in the dopamine system, such as the dopamine receptors genes for dopamine receptors type 2, 3, and 4 (*DRD2*, *DRD3*, and *DRD4*), the dopamine transporter (*DAT1*) gene, and enzymes involved in dopamine degradation (catechol-o-methyl-transferase, (COMT)) have also a link with addiction and obesity and have been discussed in other review articles (Lindgren et al., 2017; Sanna et al., 2020; Wang et al., 2019).

Despite these early addiction genetics findings, it was not until the GWAS method's emergence that geneticists could identify novel associations and provide new genes of interest as potential addiction treatment targets. This method performs statistical analyses on many polymorphisms throughout the whole genome, solving the inherent bias problem of analyzing only the hypothesis-driven candidate-genes with known or suspected connections to the addiction endophenotype of interest (Crist et al., 2019). Based on factor analysis of the Diagnostic and Statistical Manual of Mental Disorders (DSM) symptoms, GWAS for substance use disorders produced statistically robust and clinically interesting results. Detailed meta-analyses for candidate gene studies and GWAS for alcohol, tobacco, cannabis, cocaine, and opioids are discussed in another review that includes 150 meta-analyses for SUD (Lopez-Leon et al., 2021). For example, the earliest and most relevant findings with clinical relevance derived from GWAS studies related to nicotine and alcohol addiction (Hancock et al., 2018). Thus, GWAS results of nicotine dependence revealed genetic variations in nicotinic acetylcholine receptor subunit genes such as *CHRNB3, CHRNA6,* and enzymes involved in nicotine metabolism *CYP2A6 and CYP2B6* (Furberg et al., 2010; Hancock et al., 2015; Thorgeirsson et al., 2010). GWAS meta-analyses identified targets in chromosome 15q and 20q, in which the cholinergic receptor nicotinic alpha genes (CHRNA) are located. The SNPs identified in several GWAS were located in the CHRNA3, CHRNA4, CHRNA3, DBH (dopamine beta-hydroxylase), and PEX2 genes. Besides, candidate gene studies revealed the strongest associations with the dopamine receptor 2 gene (DRD2), the galanin receptor 1 gene (GALR1), and with tetratricopeptide repeat domain 12 (TTC12) (Lopez-Leon et al., 2021). Interestingly, the cholinergic receptor nicotinic subunit CHRNA2 has been implicated with cannabis use disorder (Demontis et al., 2019). A recent GWAS of nicotine metabolism and cigarette consumption, measured in current smokers of European

descent, showed a fine-mapping of chromosome 19 revealing putatively causal variants mapping to CYP2A6, MAP3K10, ADCK4, and CYP2B6, in addition to a novel chromosome 4 region mapping to TMPRSS11E and several UGT2 genes (Buchwald et al., 2020). The authors identified SNPs associated with nicotine clearance estimated and metabolism and identified novel loci influencing the nicotine metabolism and tobacco exposure phenotypes. Studies on alcohol use disorder showed genes associated with alcohol pharmacokinetics, such as alcohol dehydrogenase (*ADH1B-ADH1C*) and aldehyde dehydrogenase (*ALDH2)* as well some other genes linked with regulatory protein functions, such as the *SERPINC1, GCKR, SGOL1, KLB, AUTS2,* and *TF* (Clarke et al., 2017; Takeuchi et al., 2011; Way et al., 2015). Other GWAS studies showed more neuropsychiatric candidate genes identified, such as the GABA transporter 1 (SLC6A1) and Adrenoceptor Alpha 2A (ADRA2A) region (Adkins et al., 2015). In addition, candidate gene studies showed associations that were protective against alcohol use disorder, including the alcohol dehydrogenase genes (ADH1B, ADH1C) and the aldehyde dehydrogenase 2 (ALDH2). Other genes significantly associated with alcohol dependence and alcohol use disorder were three dopamine-related genes (DRD2, DRD4, and SLC6A3), serotonin-related genes (SLC6A4, HTR2A), GABA receptor genes (GABRA2 and GABRB2), an opioid receptor gene (OPRM1), and the tumor necrosis factor (TNF) (Lopez-Leon et al., 2021). Significant variants from GWAS of opioid use disorder were associated with genes encoding potassium voltage-gated ion channels, such as the *KCNG2* and *KCNC1* (Crist et al., 2019). A recent meta-analysis of GWAS revealed that the HIST1H2BD gene was associated with cocaine dependence, a gene located in a region on chromosome 6, which has been previously associated with schizophrenia (Cabana-Domínguez et al., 2019). Regarding food addiction, only one GWAS study provided results of genetic variations to our knowledge. An enrichment for gene members of the MAPK

signaling pathway was revealed, but no candidate SNP coincident for substance use disorder was significantly associated with food addiction due to a possible limited study power (Cornelis et al., 2016). Therefore, some studies have the limitation of insufficiently powered GWAS sample sizes for complex traits tested, and the GWAS-identified SNPs only explain a small proportion of the phenotypic variance to develop the addictive behavior emphasizing the polygenic multifactorial component of addiction (Martin et al., 2019; Vink, 2016). Indeed, GWAS studies revealed that most SNPs are produced in non-coding sequence, changing RNA expression and DNA methylation, ultimately altering gene activity. This evidence highlights the importance of understanding the cross-talk between genetics and epigenetics in the individual susceptibility to addiction since this complex disease is mediated by the interplay between genetic and epigenetic factors in many cells (Maurano et al., 2012).

Recent studies have recognized the importance of gene interaction complexity and integrate DNA, RNA, and protein sequence data with GWAS findings (Hancock et al., 2018). Similarly, other investigations considered the simultaneous involvement of multiple genes in the regulation of pathways underpinning addiction. Remarkably, a study used the powerful multilocus genetic profile method, a combined genetic index reflecting the effect of numerous polymorphic loci functionality relevant to a specific neurobiological mechanism (Nikolova et al., 2011). The polygenetic risk score method uses entire GWAS datasets, with several million loci, and has been proposed as a complementary method to investigate complex traits' polygenetic architecture by considering a large set of SNPs simultaneously (Lewis and Vassos, 2020). In this method, a GWAS is conducted on an initial discovery sample, and the p-values of SNPs are obtained. Subsequently, an independent target sample is analyzed by constructing a polygenic risk score consisting of each subject's associated

alleles' weighted sum. This polygenic risk score has been used to predict individual genetic liability for drug addiction (Barr et al., 2020; Chen et al., 2018; Frank et al., 2012; Vink et al., 2014). Few studies have been developed using this method in addiction. This method allows the inclusion of several polymorphisms with minor independent effects but with jointly significant effects. A multilocus genetic profile represents the cumulative impact of functional polymorphisms on dopamine signaling, comprising *COMPT, DAT*, *DRD2,* and *DRD4* genes, which individually produce variation in striatal dopamine signaling, that can explain individual differences in reward-related ventral striatum variability (Nikolova et al., 2011). A study employing this genetic methodology to food addiction found a significantly increased dopamine signaling in the food addiction group compared to controls supporting a reward-based causal model progressing from an inherent biological susceptibility to increased risk for overeating, and ultimately to develop addiction to hyperpalatable food (Davis et al., 2013). Another study described variations in a multilocus genetic score related to dopamine signaling associated with sugar consumption differences in children with intrauterine growth restriction, suggesting that dopamine function is involved in these children's behavioral features (Silveira et al., 2018).

3. Epigenetics of vulnerability and resilience

The fact that not all individuals who consume drugs develop addiction suggests the existence of individual resilient and vulnerable factors that contribute to addiction's pathogenesis and persistence. Experiences in life and environmental factors can shape epigenetic profiles that may modify individuals' vulnerability to addiction. Epigenetic mechanisms are a set of

posttranslational modifications that play an essential role in changing the brain for a lifetime, although they can also be reversible and transgenerationally transmitted. The diversity of epigenetic modifications described in addiction due to exposure to drugs of abuse include histone posttranslational modifications, DNA methylation, and changes in miRNAs (Figure 3).

Modification of histones is one of the most frequently studied epigenetic alterations in addiction. Histones are essential proteins that wrap DNA in the nucleus and condense it into chromatin. The basic building block of chromatin, also called a nucleosome, contains 147 DNA base pairs wrapped throughout an octamer comprising two copies of H2A, H2B, H3, and H4 histones (Andrews and Luger, 2011). These proteins can undergo several posttranslational modifications in which different functional groups are covalently added to amino acid residues of the N-terminal tails. Some of these modifications are acetylation, phosphorylation, and methylation, among others (Walker and Nestler, 2018). Acetylation of histone occupancy on lysine residues decreases electrostatic interaction between DNA and histone proteins to make DNA more accessible to transcriptional regulators (Kouzarides, 2007). Histone phosphorylation can occur on serine, threonine, and tyrosine residues and is generally associated with transcriptional activation (Rossetto et al., 2012). On the other hand, histone methylation occurs on lysine, and it is associated with either repression or activation of gene expression, depending on the number of methyl residues added (Kouzarides, 2002). Histone lysine acetylation tends to be protective and leads to adaptive behaviors (Hitchcock and Matthew, 2014). Histone lysine acetylation is continuously fluctuating throughout the brain after exposure to different drugs of abuse, including cocaine (Rogge et al., 2013), nicotine (Huang et al., 2014), morphine (Mashayekhi et al., 2012), heroin (Egervari et al., 2017), and amphetamine (Renthal et al., 2008), among others. Specifically, histone

deacetylases 3 negatively regulates cocaine-induced conditioned place preference acquisition (Rogge et al., 2013). Concerning nicotine, it has been shown that the application of a histone deacetylation inhibitor can mimic the priming effects of nicotine (Huang et al., 2014). With morphine, there are tissue and promoter-specific histone modifications around the BDNF gene's individual promoters in response to chronic morphine and withdrawal (Mashayekhi et al., 2012). Heroin use leads to hyperacetylation of histone H3 in the dorsal striatum that produces glutamate transcription changes (Egervari et al., 2017). Studies performed with chronic amphetamine administration showed increased histone H3 methylation on the c-fos promoter and increased expression levels of the H3 histone methyltransferase, KMT1A (lysine methyltransferase 1A, formerly SUV39H1) (Renthal et al., 2008). In the last years, histones' acetylation and phosphorylation have been widely studied using candidate gene approaches in response to cocaine (Rogge et al., 2013; Sadri-Vakili, 2015). These studies confirm that acute or repeated exposure to some abusive drugs can increase H3 and H4 acetylation in several brain areas involved in the reward circuitry. Nevertheless, more studies are needed to precise their specific roles in addiction physiology.

Histone phosphorylation is another posttranslational modification usually observed in substance use disorder. Drugs of abuse and natural reinforcements promote the nuclear accumulation of dopamine- and cAMP-regulated phosphoprotein DARPP-32. The nuclear accumulation of DARP-32 leads to an increase in H3 histone phosphorylation (Stipanovich et al., 2009). Another common histone modification is lysine methylation. Histone lysine methylation is particularly complex and variable. Repeated exposure of drugs, such as cocaine and morphine, reduces global levels of histone methylation marks (H3K9me2 and H3K9me3) in the NAc (Sun et al., 2012). On the other hand, methamphetamine exposure decreases H3K27me2 levels but increases H3K4me2 and H3K4me3 levels in the NAc

(Aguilar-Valles et al., 2014). These studies are prototypical and suggest that histone methylation regulation in response to drugs of abuse is complex and drug- and region-specific. Correlations of altered transcript levels with activating and repressive marks of methylation have also been investigated after cocaine exposure. Exonic enrichment of H3K4me1, H3K4me3, H3K36me3 were positively correlated with transcriptional levels, whereas H3K9me2, H3K9me3, and H3K27me3 were negatively correlated (Feng et al., 2014), suggesting novel regulation modes on the epigenome in the NAc by which cocaine alters the brain. Identifying drug-induced transformations in histone acetylation, phosphorylation, and methylation in the NAc and other brain areas suggests that these modifications might involve drug addiction regulation. More studies are needed to propose a molecular mechanism to explain these adaptations after exposure to the different drugs of abuse. However, evidence suggests that the combination of inherited predispositions, environmental stimuli, and exposure to addictive substances leading to histone modifications may represent protective and risk factors in developing addiction.

It has been recently shown that dopamine is associated with chromatin to form a previously unknown epigenetic regulation called 'dopaminylation.' Rats undergoing withdrawal from cocaine showed an accumulation of histone H3 glutamine 5 dopaminylation (H3Q5dop) in the VTA. Consequently, intra VTA viral manipulations were produced to induce decreasing levels of H3Q5dop. This manipulation prevented cocaine-mediated gene expression changes and cocaine-seeking behavior in rats during withdrawal (Lepack et al., 2020). This previously uncharacterized chromatin modification opens a new research window in the underlying substance use disorder's epigenetic regulation mechanisms.

In summary, drug use may produce enduring alterations in gene expression via histone modifications to individuals carrying susceptibility genes exposed to adverse environmental

factors. These post-translational transformations may influence susceptibility to addictive disorders. Enhanced vulnerability to abuse drugs can give feedback at an increased risk of future drug consumption, leading to further modifications on the epigenome and gene expression (Wong et al., 2011).

DNA methylation is a stable epigenetic mark that occurs with the covalent modification of DNA by DNA methyltransferases. In this modification, DNA methyltransferases trigger the addition of a methyl group to cytosine-phosphoguanine (CpG) at the C5 position (5-mC) (Bird, 2002). DNA methylation in gene promoters is associated with repression, whereas DNA methylation in gene bodies is associated with active transcription (Nestler and Lüscher, 2019). Repression of DNA by DNA methylation can be detrimental and enhance the risk to develop addiction and depressive-like behaviors (Hitchcock and Matthew, 2014). However, little is known about the specific impact of DNA methylation in protective and risk factors in substance use disorder, and most studies focus on the specific effect of drugs of abuse in the expression of DNA methyltransferases. Methamphetamine self-administration paired with electrical footshocks resulted in differential DNA hydroxymethylation and increased expression of potassium channel mRNAs in the NAc of those rats that showed a resilient phenotype against the adverse consequences associated with methamphetamine (Cadet et al., 2019). Subchronic methamphetamine treatment-induced different patterns of DNA methyltransferase 1 mRNA expression in the nucleus caudatus and NAc in rats, which increased DNA methylation (Numachi et al., 2007). Other studies showed that repeated cocaine exposure increased the expression of methyl-CpG binding protein 2 and produced de novo DNA methylation (Bodetto et al., 2013). By contrast, nicotine microinjections in the mouse cortex and hippocampus decreased mRNA and DNA methyltransferase 1 expression

levels but increased glutamic acid decarboxylase 67 (GAD_{67}) expression levels in the frontal cortex (Satta et al., 2008).

Moreover, a recent study in alcohol use disorder subjects revealed that chronic alcohol drinking results in increased DNA methylation of NR3C1 exon variant 1H, associated with reduced NR3C1 mRNA and protein expression levels in PFC and other cortico-limbic regions (Gatta et al., 2019). Oxycodone also produced demethylation of genes implicated in synaptic plasticity (synaptophysin and post-synaptic density protein 95) in the VTA of rats, which can be prevented with oxytocin pretreatment (Fan et al., 2019). However, a global mapping of DNA methylation changes in the brain at single-nucleotide resolution in response to abuse drugs is still not available. The generation of DNA methylation genome-wide maps would allow a better understanding of the role of this epigenetic mark in substance use disorder.

The study of epigenetic marks is currently moving one step forward due to the latest technological advances. The combination of next-generation sequencing (NGS) and chromatin immunoprecipitation (ChIP) has now provided novel descriptive information about the presence of epigenetic marks involved in addiction and other neurological diseases. Even though this information is of great importance, the real revolution relies on the technology that enables the induction of discrete epigenetic marks in brain cells. This technology is termed "neuroepigenetic editing". Before its existence, experiments in animal models were based on the over-or underexpression of related epigenetic enzymes (as histone or chromatin modification enzymes), which target hundreds of genes and, therefore, have undesirable off-target effects and unpredictable compensatory consequences. In contrast, neuroepigenetic editing techniques, produce single and locus-specific epigenetic modifications, thus allowing to establish a causal relationship between the epigenetic mark

and its consequence. There are three main strategies to produce discrete neuroepigenetic editions: zinc-finger proteins (Heller et al., 2014), transcription activator-like effectors (Heller et al., 2014), and clustered regularly interspaced short palindromic repeats CRISPR/Cas9. Among them, CRISPR/Cas9 stands out due to the simplicity of its design, the low price of the synthesis, the scalability, versatility, and most importantly, the cell specificity and temporal precision. For instance, CRISPR/Cas9 has been used to target methyl-CpG-binding proteins (Swiech et al., 2015), and Cas9 has also been used after its fusion with DNA methylating enzymes to edit the methylation profile of specific enhancers and promoters (Liu et al., 2016). CRISPR/Cas9 applications in the mammalian nervous system exceed this manuscript's aim and have been deeply reviewed before (Day, 2019; Savell et al., 2019). Therefore, neuroepigenetic editing, although recent, is proving to be a very promising field in basic and translational research.

In the food addiction field, the formation of reward-related associative memories in rats upregulated essential plasticity genes in the VTA correlated with memory strength and associated with gene-specific changes in DNA methylation (Day et al., 2013). Food addicted mice also showed a significant decrease in DNA methylation of the *CNR1* gene promoter in the PFC, which was associated with an upregulation of CB1 protein expression in the same brain area (Mancino et al., 2015).

Thus, epigenetic signatures have an essential role in vulnerability or resilience to addiction. The epigenetic mechanisms involve modifications in the stable-state expression levels of a set of genes and produce 'latent' changes in other genes' inducibility, activation, and inactivation (Nestler, 2014). Regulation of gene inducibility can be seen as "latent" in that it would not be evident by analysis of mRNA or protein levels. For instance, cocaine induces latent effects by changing the chromatin structure at many genes, which alter their

inducibility in response to a subsequent stimulus. These latent changes can determine an individual's vulnerability to addiction later in life upon drug exposure or affect relapse in long-term abstinent individuals after re-exposure to the drug. However, drugs or other environmental exposures can also induce transgenerational epigenetic inheritance of addiction vulnerability by epigenetic changes in germinal cells, which are then passed on to progeny and alter their vulnerability to addiction (Meaney and Szyf, 2005).

4. miRNAs of vulnerability and resilience: alteration of miRNA expression as a novel therapeutical tool

Non-coding RNAs that do not translate into proteins play an essential role in cell function. Among these, miRNAs are of particular relevance for cell biology and are related to many processes, including addiction.

miRNAs are short RNA transcripts with around 22 nucleotides that are endogenously expressed by cells to regulate gene expression post-transcriptionally in a very dynamic way (Kenny, 2014). The identification of more than 2,000 miRNAs in the human genome suggests high conservation through evolution (Alles et al., 2019). Each one can impact the post-transcription of thousands of protein-coding genes, eliciting a rising interest among the scientific community in most diverse fields, including addiction.

miRNAs are transcribed by RNA polymerase II in their native form and processed into so-called pri-miRNAs. Pri-miRNAs are processed by the Drosha enzyme into pre-miRNAs of around 70 nucleotides and exported from the nucleus. Outside of the nucleus, they are further processed by the Dicer enzyme into a duplex RNA (dsRNA). The protein family Argonaute plays a crucial role in RNA silencing, being a component of the RNA-induced silencing

complex (RISC). Together with Dicer and Argonaute, the dsRNA incorporates into RISC. RISC is responsible for the gene silencing phenomenon known as RNA interference (RNAi). Argonaute proteins bind different classes of small non-coding RNAs, including microRNAs (miRNAs). One RNA strand is removed from the dsRNA molecule, and the other RNA remains retained in the RISC as a mature molecule of miRNA (Figure 4). Ago 2 (one of the four members of the Argonaute family) has endonucleolytic activity, identifies the target mRNA-miRNA complementary sites, and degrades the target mRNA (Liu et al., 2004). However, miRNAs can also block mRNA translation by preventing mRNA interaction with ribosomes and targeting mRNAs to processing bodies for degradation (Filipowicz et al., 2008). In addition to these "canonical" miRNA roles in the cytoplasm, some findings have described miRNA targeting different cell compartments such as the nucleus and the cytoplasm and newer functions developed by miRNAs in these locations. These miRNAs can relocate to the nucleus, alter mRNA stability in the nucleolus, and affect alternative splicing (Catalanotto et al., 2016). In neurons, miRNAs develop specific functions in dendrites and can be secreted to synapsis in exosomes (Smith and Kenny, 2018). When miRNAs bind to the 3'UTR of mRNAs, they affect mRNA translation and stability and mRNA distribution in dendrites and synapsis (Most et al., 2014).

miRNAs play a role in synaptic plasticity events closely related to addiction development (Smith and Kenny, 2018). Synaptic plasticity processes, such as enlargement of dendritic spines, changes in AMPA/NMDA receptor ratio, and activation of metalloproteases, are related to opiates, nicotine, and cocaine relapse (Smith and Kenny, 2018). miR-132 and miR-212 are two examples of miRNAs with essential functions in synaptic plasticity and dendritic growth through the interaction with the brain-derived neurotrophic factor (BDNF). Inhibition

of miR-132 reduces the number of mushroom-like spines while immature filopodia increases, whereas the deletion of miR-132 and miR-212 decreases dendritic length and complexity in the neurons of the hippocampus (Smith and Kenny, 2018). miR-132 and miR-212 are upregulated in the striatum after cocaine administration (Sadakierska-Chudy et al., 2017) and upon cocaine reinstatement (Quinn et al., 2015).

Alongside the increasing knowledge about miRNAs, the technology required to alter miRNA expression moves forward. Inhibition and overexpression of miRNAs are now possible by specific experimental approaches, therefore becoming promising therapeutic tools. As miRNAs eventually achieve their target mRNA's degradation, their suppression would correlate with their target mRNAs' overexpression or an increased translation. The opposite would happen when overexpressed with downregulation of their target mRNAs or a decreased gene expression. Most strategies to date that aimed to inhibit miRNAs consist of delivering a DNA vector encoding for the RNA-based inhibitory molecule, thus providing a long-lasting expression. *In vitro* studies have demonstrated that locked nucleic acids (LNA) are also very potent tools. They consist of a short synthetic RNA oligonucleotide containing a modified nucleotide, which sharply increases its stability (Papargyri et al., 2020; Rasmussen and Roberts, 2007). The use of LNA for the knockdown of miRNA is beneficial because these probes have a high affinity for their small RNA targets and excellent mismatch-discrimination capability (Rasmussen and Roberts, 2007). The structurally most straightforward approach for miRNA inhibition *in vivo* is antagomiR. It consists of a single-stranded RNA molecule entirely complementary to the target miRNA that hybridizes and prevents the miRNA's union to an mRNA molecule (Scherr et al., 2007). This useful technology is far from being optimal, as antagomiRs are unstable and degraded quickly. To

overcome this limitation, "Sponges" have been generated with up to eight regions antisense to target miRNAs located tandemly in the same transcript. (Ebert et al., 2007). Sponges transcribed by RNA Pol II can target different miRNAs from the same family. However, one of the most potent approaches for miRNA inhibition strategies is the Tough Decoy inhibitor (TuD) (Bak et al., 2013; Hollensen et al., 2018). TuDs are 100 nucleotides long and form a hairpin structure that increases their stability and contains an unpaired region in the middle. Both strands from this unpaired region are complementary to the target miRNA. TuD technology was first described by expressing them through RNA Pol III (Haraguchi et al., 2009). However, TuDs can also be transcribed by RNA Pol II, which confers higher tissue specificity (Bak et al., 2013). The latest advances show the possibility of targeting two different miRNAs with the same TuD construct and forming TuD clusters to increase the number of miRNA-binding sites (Hollensen et al., 2013). These novel inhibitors (Sponges and TuDs) may be further improved by the insertion of a short region (4 nucleotides) of self-complementary nucleotides that form a "bulge." "Bulge" makes inhibitors less likely to be degraded by the Argonaute complex after miRNA pairing (Ebert et al., 2007; Yoo et al., 2017).

Interestingly, circular RNAs (circRNAs) were discovered to be naturally expressed by cells from most tissues. One well-known circRNA is ciRS-7 that contains 70 times the complementary sequence for a miRNA termed miR-7. ciRS-7 actuates as a "sponge" by binding miR-7, and its circular structure makes it more resistant to exonucleases (Hansen et al., 2013). Recently, other endogenously expressed circRNAs have been described to different sponge miRNAs, opening the door for more miRNA inhibition possibilities for

therapeutic purposes (Li et al., 2020). Methods for *in vitro* and *in vivo* generation of circRNAs have successfully been developed (Ebermann et al., 2020).

The novel technology required to alter miRNAs expression has potential therapeutic utility. The capacity to be secreted extracellularly and to the systemic circulation has led miRNAs to be considered potential biomarkers for diagnosing and preventing addiction. They can constitute relevant indicators of a resilient or vulnerable phenotype to addiction. The technology required to alter miRNA expression is dynamically progressing, becoming promising therapeutic tools in the field of addiction.

6. Future directions

Recent research advances have allowed a better understanding of the genetic and epigenetic bases underlying addiction development. The interaction between multiple environmental factors and polygenetic influences is responsible for the development of this disease. Multiple gene variants have been reported to participate in the individual vulnerability to develop addiction. Several of these variants were initially identified by using gene candidate approaches. However, GWAS's more recent use has provided a rapid advance in these genetic mechanisms' knowledge. The recent technological improvements in these GWAS approaches would provide definitive progress for better understand the complex interactions among the multiple genes involved in the pathogenesis of addiction. Innovative genomic approaches now combine animal and human studies for cross-talks and validation of the data, which mainly improves the identification of the functional relevance of the candidate genes identified.

The recent advances in the knowledge of the complex mechanisms involved in the epigenetic control of gene expression and the novel technological tools to manipulate these mechanisms will allow us to definitively understand the capability of environmental factors to modify gene expression and their influence on the development of the addictive processes. Identifying these epigenetic factors opens new possibilities to define more precise biomarkers of vulnerability and resilience to addiction and may open innovative therapeutic perspectives that are still unexploited.

All these genetic and epigenetic tools are now available. The use of these novel tools in creative and well-designed experimental approaches would undoubtedly allow in a near-future definitive advance to clarify the involvement of these mechanisms in addiction development. This knowledge will be crucial to develop new therapeutic strategies for better management of addict patients.

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Legends of the figures

Figure 1. Addiction has a multifactorial inheritance pattern with an interaction between different gene networks and multiple environmental factors that strongly impact brain function both during development and throughout adulthood, influencing behavior.

Figure 2. In the multifactorial addiction model, the genetic factor plays an essential role in the vulnerable or resilient individual susceptibility to developing this brain disease. In this model, the individuals' phenotypes follow a normal distribution. The majority are located in the center of the inverted U-shaped curve. The minorities represent the extremes subpopulations of a resilient or vulnerable quantitative continuum of a phenotype where some individuals can pass a certain threshold and become affected.

Figure 3. Epigenetic mechanisms alter individuals phenotype. Substances of abuse, environmental factors, and life experiences can modify epigenetic marks and reshape an individual's phenotype. Histone modification, DNA methylation, and non-coding RNAs are the most common epigenetic modifications. These modifications regulate gene expression without causing variations in the DNA sequence. The N-terminals tails of histones can undergo several post-transcriptional modifications, including acetylation, methylation, and phosphorylation, among others. Histone modification can lead to either activation or repression of gene expression, depending on the residues modified and the type of modification. DNA methylation is the covalent modification of cytosine residues in CpG dinucleotides within gene sequences. Contrary to histone modification, DNA methylation is associated with transcriptional silencing. However, DNA methylation in gene bodies is linked with active transcription. Non-coding RNAs include miRNAs that regulate gene expression through post-transcriptional silencing of genes.

Figure 4. miRNA generation, maturation, and silencing mechanisms. miRNAs are transcribed by RNA polymerase II in their native form and processed into so-called pri-miRNAs. Pri-miRNAs are processed by the Drosha enzyme into pre-miRNAs and exported from the nucleus. At that point, they consist of around 70 nucleotides. The Dicer enzyme further processes them into a duplex (dsRNA) outside of the nucleus. Together with Dicer and Argonaute, the miRNA incorporates into an RNA-induced silencing complex (RISC). One RNA strand is removed, and the mature molecule of the miRNA is retained in the RISC. The miRNA silencing mechanisms comprise Locked Nucleic Acid (LNA), antagomiR, sponges, and tough decoy (TuD). LNA use for miRNA knockdown is beneficial because these probes have a high affinity for their small RNA targets and exceptional mismatch-discrimination ability. AntagomiR consists of a single-stranded RNA molecule entirely complementary to the target miRNA. They hybridize to prevent the union of the miRNA to an mRNA molecule. Sponges have up to eight regions antisense to target miRNAs located tandemly in the same transcript. Sponges, transcribed by RNA Pol II, can target different miRNAs from the same family. TuDs are 100 nucleotides long and form a hairpin structure that increases their stability and contains an unpaired region in the middle. Both strands from this unpaired region are complementary to the target miRNA. With the same TuD construct, there is the possibility of targeting two different miRNAs and forming TuD clusters to increase the number of miRNA-binding sites.