

Epigenetic Factors in Schizophrenia: Mechanisms and Experimental Approaches

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Abstract

Schizophrenia is a chronic mental disorder that is still poorly understood despite decades of study. Many factors have been found to contribute to the pathogenesis, including neurodevelopmental disturbance, genetic risk, and environmental insult, but no single root cause has emerged. While evidence from twin studies suggests a strong heritable component, few individual loci have been identified in genomewide screens, suggesting a role for epigenetic effects. Rather, large numbers of weakly acting loci may cumulatively increase disease risk, including several mapping to epigenetic pathways. In this review, we discuss mechanisms of epigenetic regulation and evidence for an epigenetic contribution to disease phenotype. We further describe the range of experimental tools currently available to study epigenetic effects associated with the disease.

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Schizophrenia occurs in about 1% of the population and is highly debilitating socially and economically [1]. The aetiology is complex and poorly understood, and the interplay of genetic and environmental factors appears to be important in disease development. Symptoms defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) are classified as “positive” or “negative” [2]. Positive symptoms (e.g., hallucinations, delusions, and disorganized thought) arise from excess expression of normal function, while negative symptoms (e.g., reduced emotional expression, loss of the ability to experience pleasure, poverty of speech, social withdrawal, and catatonic immobility) reflect a decrease or flattening of normal emotional function. It is common for schizophrenia to be comorbid with other disorders such as depression and personality disorders however, adding to the difficulty of diagnosis. Currently, clinical treatments rely mainly on antipsychotic compounds that act by the inhibition of the type 2 dopamine receptor pathway [3]. These compounds primarily relieve the so-called positive symptoms. However, they are largely ineffective for relief of important symptoms such as attention and memory defects. Unfortunately, this

fails to prevent poor personal and social outcomes for schizophrenia [4].

Schizophrenia is associated with impaired performance across a range of measurable parameters, including neuron volume and number, synaptic connectivity, neurochemical balance, and sensory gating [5]. Post-mortem studies show dysregulation of the transcriptomes and proteomes in pre-frontal and temporal cortex oligodendrocytes [6–8]. Similarly, many key proteins responsible for effective neuronal function, including ion channels and neurotransmitter pathway enzymes, show expression changes in post mortem brain from schizophrenia patients [9]. Two general themes are central to most proposed models explaining schizophrenia pathology: the role of the synapse [10] and the early stages of neuronal development (differentiation and maturation) [11].

Genetics and Epigenetics of Schizophrenia

Schizophrenia shows a high degree of heritability (~64% based on family studies) [12], leading to efforts to map causal loci using high-throughput genomics technologies. To date, this work has failed, in general, to highlight strong individual genetic risk factors. Important exceptions include a deletion at 22q11 (~25% of individuals with this deletion have symptoms of schizophrenia [13] and a number of SNPs mapping to the major histocompatibility complex [14]. However, large numbers of frequently occurring low risk variants, many mapping to early neurodevelopmental pathways have been discovered. In particular, a genome-wide association study that included 36,989 cases and 113,075 individuals led to the identification of 128 common variants in 108 different loci, each contributing marginally to risk but cumulatively accounting for a modest disease risk (OR ~1.3) [15]. Many of the identified genes are involved in glutamatergic neurotransmission, synaptic plasticity, or encode voltage-gated calcium channels, as well as the product of the *DRD2* gene (dopamine receptor D2), the main target of antipsychotic drugs. Furthermore, transcripts of these 108 genes show elevated expression patterns in fetal brain when compared to post-natal brain [16]. Similar studies have reported increased expression of schizophrenia risk genes during early brain development [17, 18]. These findings are consistent with a generalized neurodevelopmental role for many genes associated with risk of schizophrenia.

In contrast to these common risk factors, a number of schizophrenia-associated loci have been reported that are

rare in the disease population. Copy number variants mapping to 8 loci were identified in a meta-analysis of 21,094 cases and 20,227 controls, including *NRXN1*, a neurexin involved in synapse formation and neurotransmission [19]. Other loss-of-function mutations have been reported for the gene encoding histone H3 methyltransferase *SETD1A* [20]. These add to studies implicating epigenetic factors in schizophrenia, for example, the finding that schizophrenia risk loci are more likely to be found proximal to DNA methylation quantitative trait loci [21]. A recent investigation that combined a transcription-wide association study with descriptions of gene expression, splicing, and chromatin activity, found 157 genes with transcriptional changes specifically associated with schizophrenia [22]. A significant proportion of these were strongly associated with nearby chromatin features, again supporting an important role for epigenetic mechanisms in the disease. Similarly, some variants associate significantly with disease but appear to be limited to individual pedigrees. A translocation found to segregate in a Scottish pedigree with mental disorders, including schizophrenia, resulted in the disruption of the *DISC1* gene involved in synapse function and early neurodevelopment [23, 24]. However, *DISC1* was not identified as significant in the genome-wide association study mentioned above.

Mechanisms of Epigenetic Regulation

The above observations have switched research focus to a search for epigenetic factors that might explain the discrepancy between the strong inheritance of schizophrenic disease and the lack of strong genetic markers. Epigenetics broadly refers to heritable changes in phenotype that are not encoded in the DNA sequence of the genome. These changes are therefore not permanent but can be carried from parent to daughter cell (or from one generation to the next), by a form of molecular memory that regulates gene expression programs. Several mechanisms that can alter the chromatin environment surrounding the regulated genes underlie these effects.

The level of mRNA and protein expression from a gene relies not only on features of the primary DNA sequence such as promoters and ribosome-binding sites. It is now clear that dynamic changes in the structure of the surrounding chromatin allow alteration in the activation, repression, and general regulation of genes [25]. Several distinct forms of epigenetic regulation are known. These distinct mechanisms cross-talk with each other in ways

that are not fully understood, and include (a) direct methylation of DNA; (b) modification of the associated histone molecules by a range of chemical adducts (e.g., methylation, acetylation ubiquitination); (c) exchange of histone molecules with related isoforms; (d) regulation of access to DNA by manipulation of chromatin by nucleosome remodellers. While we focus here on the above mentioned pathways, it should be noted that many other mechanisms have been implicated in epigenetic effects, including those based on non-coding RNAs, non-genic DNAs, differential exosome expression, and alternative modification products catalyzed by the Ten-Eleven Translocation family of enzymes including 5-hydroxymethyl cytosine, 5-formylcytosine, 5-carboxylcytosine.

Direct methylation of DNA in mammals is mediated primarily by the DNMT (DNA methyltransferase) family that catalyzes the transfer of methyl groups to the C5 position of CpG dinucleotides. Since levels of DNA methylation can be affected by dietary restriction, a link between periods of famine and increased incidence of schizophrenia has been proposed to be at least partly based on epigenetic phenomena [26, 27]. Gene expression can be influenced by methylation of the underlying DNA in a number of ways, for example, by preventing access to transcription factors, or by recruiting chromatin-modifying enzymes. Much of the work toward epigenetically characterizing the schizophrenia epigenome has involved measuring direct DNA methylation through bisulphite sequencing and similar methods. For example, a recent study (41 cases, 46 controls) across 4 brain regions found many genome regions that displayed differentially methylated DNA [28], while a larger study (689 cases, 645 controls) using blood samples similarly identified many differentially methylated loci [29]. Other studies support the importance of DNA cytosine methylation in schizophrenia [30–32]. Differential DNA methylation studies to date have mapped to major neurotransmitter pathways. For example, promoters of the reelin (RELN) and glutamic acid decarboxylase (GAD1) genes, components of the γ -aminobutyric acid-ergic pathway, are hypermethylated in schizophrenia [33, 34], while the promoter for the catechol-O-methyltransferase gene, a component of the dopaminergic pathway, was found to be hypomethylated in postmortem schizophrenia brains [35]. The serotonin pathway has also been implicated, with hypermethylation of the promoter of the serotonin receptor type-1 gene (HTR1A) gene being reported for blood samples of schizophrenia patients relative to controls [36]. Additionally, while not reviewed here, the effect of antipsychotics on DNA methylation is now widely reported (for recent review see [37]).

Post-translational modifications (PTMs) of histone proteins also have a significant effects on epigenetic regulation of neuropsychiatric disorder, and schizophrenia [38]. Each nucleosome comprises 2 copies each of 4 histone proteins (H2A, H2B, H3, H4). The N-terminal tails of H2A, H2B, H3 and H4 histones extend from the globular regions of the histone structure and are subject to extensive and dynamic modifications. Over 70 distinct histone amino acid modifications have been described, including methylation, phosphorylation, ubiquitination, acetylation and sumoylation [39]. They contribute to the regulation of gene expression in several ways, none completely understood. One mechanism involves the recruitment (or displacement) transcription activators or repressors to the cognate gene through enhanced protein interaction affinity, while another mechanism involves chromatin conformational changes that increase the accessibility for positive and negative regulatory factors. Some PTMs at specific amino acids are associated with repression of gene expression (e.g., methylation of histone 3 at lysines 9 and 27), while others are associated with activation (e.g., acetylation at histone 3 at lysines 9 and 14). The observation that distinct combinations of histone PTMs are found in different transcriptional and genomic contexts led to the proposal that a “histone code,” capable of interpretation by the transcription machinery, governs the expression of associated genes [40].

These PTMs are regulated by a network of enzymes (e.g., histone methyltransferases and histone demethylases) that are often housed in multi-subunit protein complexes. For example, the Polycomb Repressor Complex (PRC1, PRC2) family of complexes mediate gene silencing via generation of trimethylated lysine 27 on histone H3 (H3K27me3) and ubiquitination of lysine 119 of histone H2A (H2AK119Ub). Similarly, the COMPASS family is associated with activating histone marks (H3K36me3). These complexes typically contain 5–10 protein subunits, including a core enzyme responsible for the histone modification reaction (e.g., EZH2 in PRC2; SETD1A in COMPASS). The functions of the additional proteins in these and similar complexes are under intense study, but typically include modulation of the core enzymatic activity, targeting of the complex to specific genomic loci, and mediating interactions with other chromatin and signaling proteins.

Another class of chromatin modification enzymes, termed “remodellers” is capable of altering the structure of the chromatin itself in order to enhance or inhibit local gene expression. While different remodeling complexes employ different mechanisms, they share an ATPase-

translocation function that can manipulate the histone-nucleosome architecture [41].

Relatively few reports have been published to date that specifically address the role of individual epigenetic pathways in schizophrenia. This can largely be attributed to the highly specialized technologies needed to study these effects, and to the difficulty in obtaining precious samples. Instead, the focus has been on assessing the levels of a small number of histone PTMs and the balance between histone acetylation and deacetylation. The use of peripheral blood mononuclear cells is one way to obtain suitable samples. Studies on histone PTM levels in schizophrenia found increased levels of the repressive histone mark H3K9me2 that was correlated with the age of onset, as well as resistance to treatment when this position was acetylated [42]. Other workers have focused on measuring the relative abundance of the enzymes responsible for generating these histone marks. Increased histone deacetylase activity (HDAC1) in schizophrenia patients has been noted in several studies, including those in the pre-frontal cortex and the hippocampus and medial temporal lobe [43]. In keeping with this, over-expression of HDAC1 in mice is associated with behavioral abnormalities and working memory deficits [44]. HDAC2 has also been linked to schizophrenia, an effect conferred at least partly through regulation of the metabotropic glutamate receptor [45]. Finally, in an example of the emerging type of experiment now possible, analysis of enriched DNA motifs following chromatin profiling of pre-frontal cortex samples found evidence for the involvement of the MEF2C transcription factor in schizophrenia risk [46].

Environmental insult is now thought to be a major contributor to epigenetic change [47]. Maternal behavior is associated with altered histone acetylation, in addition to increased DNA methylation [48]. Similarly, mice exposed to social defeat or isolation show increased levels of repressive histone marks, such as methylation of histone H3 at lysines 9 and 27. Interestingly, these effects are reversible by administration of the antidepressant imipramine [49], while deacetylation of histones in the hippocampus using overexpression of HDAC5 blocks this effect [50]. In fact, histone deacetylation inhibitors show antidepressant effects in animal models of depression [51]. Immunological stress in the form of viral infection (pre- or post-natal) may also contribute to risk of schizophrenia [52].

The connection between schizophrenia risk and epigenetic effects has led to speculation that drugs acting on epigenetic pathways may be beneficial. For example, some studies in animal models suggest that the fear ex-

tion response can be manipulated through modulation of HDAC [53]. However, the exact relevance to schizophrenia, and issues concerning which brain regions are involved, and which specific HDAC isoforms should be targeted, make general conclusions problematic.

Experimental Methods for Investigating Molecular Epigenetic Effects

The commercial availability of very high mass accuracy instruments in the last decade has led to improvements in quantitative proteomics studies, as well as increased confidence in molecular assignments when identifying proteins and PTMs. For example, acetylation and trimethylation of lysine, 2 critical epigenetic modifications on histones that are associated with different transcriptional outcome, differ by less than 0.04 Da. However these 2 modifications can be resolved using Orbitrap type instruments. In general, mass spectrometry analysis of histones can be described as “top-down” (where the intact protein is ionized and analyzed), or “bottom-up” (where the protein is first digested into peptides using a protease such as trypsin). Top-down approaches offer the possibility of a complete view of the individual histone molecule, including the exact state of PTMs present at a particular time. The drawbacks of the top-down approach are that it requires relatively specialized equipment and software, and that it needs large amounts of very pure sample [54]. A compromise approach, termed “middle-down,” involves analysis of long peptide fragments has shown considerable success [55]. Using the bottom-up approach, the very complex mixture of peptides (typically 1,000’s of distinct peptide molecules) produced following enzyme digestion are separated online reverse phase liquid chromatography (i.e., HPLC interfaced directly with the electrospray source on the mass spectrometer) [56, 57].

These approaches are particularly challenging for histone analysis for 3 reasons. First, the combinatorial arrangements of acetylation and methylation at distinct residues on a single peptide results in many isobaric molecular species. Second, histone proteolysis products tend to be hydrophilic, requiring adaptation of the HPLC gradients. Third, histones are enriched in the basic residues that are cleaved by tryptic enzymes and so produce small peptides that are difficult to analyze using mass spectrometry (and that lose valuable information concerning co-occurrence of neighboring histone marks). Generally, these obstacles are overcome through use of the enzyme

Table 1. Affinity-based chromatin enrichment techniques used in epigenetic studies

Abbreviation	Alternative name	Method
ChIP-chip	ChIP-on-chip	Chromatin immunoprecipitation of DNA using epigenetic mark-specific antibodies, followed by the identification of the associated DNA using microarrays
ChIP-seq	ChIP-sequencing	Chromatin immunoprecipitation of DNA using epigenetic mark-specific antibodies, followed by the identification of the associated DNA using sequencing
MeDIP-seq	Methylated DNA immunoprecipitation	Immunoprecipitation of methylated DNA using methylated DNA-specific antibodies, followed by the identification of the associated DNA using sequencing
4C-seq/HiC-seq	Circular chromosome conformation capture-sequencing	Cross-linking of locally interacting DNA regions, followed by the identification of those DNAs using sequencing
ChIP-MS	ChIP-mass spectrometry	Chromatin immunoprecipitation of DNA using epigenetic mark-specific antibodies, followed by the identification of the associated proteins using mass spectrometry

Arg-C (which does not cleave after lysine residues), or by using alkylation chemistry [58]. The 2 main forms of alkylation of histones involve the use of propionic acid or acetic anhydride (the later generally in deuterated form to permit naturally occurring acetylation to be distinguished from the chemically introduced form) [56, 59]. A bonus of the chemical alkylation approach is that it alters the hydrophilic properties of many modified histone peptides, allowing chromatographic separation (and quantitation) of several isobaric peptide pairs that would otherwise be indistinguishable [60].

For many histone marks, relative abundance can therefore be compared when carefully controlled MS runs are carried out, by calculating the parent ion signal (MS1) for the corresponding peptide. This generally requires manual analysis of the mass spectrometry data using instrument-specific software to obtain extracted ion chromatographs, although recently developed programs such as MaxQuant [61] and Skyline [62] have considerably reduced the workload involved. In general, label-free methods can be employed, but where possible, metabolic labeling can be used to improve resolution [63]. Unfortunately, few cell culture models are available in schizophrenia research so metabolic labeling is usually not an option.

Characterization of the enzyme complexes mediating histone PTM deposition and removal also relies heavily on mass spectrometry. These studies range from analysis of the global chromatin proteome [64], to identify of the

components of purified histone modifying protein complexes following affinity purification [65]. Recent developments include the use of affinity-tagged peptides to isolate histone PTM “readers” [66] and efforts to develop locus-specific recovery of histones using tagged nucleic acids or CRISPR-based reagents [67, 68]. Mass spectrometry also lends itself to the analysis of other epigenetic phenomena, for example, the presence of histone variants. The histone 3 variants H3.1 and H3.3, for example, can be distinguished from each other by a single amino acid difference in the N-terminal tail [69]. A family of related approaches relies on affinity purification of epigenetically marked DNA followed by analysis of the associated nucleic acid or protein by relevant techniques. These methods are generally referred to as chromatin immunoprecipitation and incorporate a capture step using antibody-based reagents. Generally, these are high-quality reagents, although concerns have been raised about the specificity of these approaches in some cases [70]. In retrospect, this is perhaps unsurprising/since in molecular terms, the difference between epigenetic marks (whose presence or absence can confer major differences in expression of the associated genes) can be subtle, for example, mono-, di-, or trimethylated lysines. Examples of these techniques are outlined in Table 1. Typically, large amounts of starting material are needed, and factors such as cell heterogeneity can cause problems.

In conclusion, emerging technologies now enable the study of several aspects of epigenetic effects in schizo-

phrenia and related pathologies. To date, these advanced approaches have not directly been applied to schizophrenia research, partly because they generally require specialized equipment and expertise, and partly due to an absence of experimental models and access to clinical material. In future, however, these approaches are likely to offer exciting new viewpoints into the role of epigenetic mechanisms in the disease.

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