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Understanding the genetic liability to schizophrenia through the neuroepigenome

John F. Fullard[1], **Tobias B. Halene**[1,4], **Claudia Giambartolomei**[1], **Vahram Haroutunian**[1,3,4], **Schahram Akbarian**[1,3], and **Panos Roussos**[1,2,4]

[1]Department of Psychiatry, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[2]Department of Genetics and Genomic Science and Institute for Multiscale Biology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[3]Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[4]Mental Illness Research, Education, and Clinical Center (VISN 3), James J. Peters VA Medical Center, Bronx, NY, USA

Abstract

The Psychiatric Genomics Consortium-Schizophrenia Workgroup (PGC-SCZ) recently identified 108 loci associated with increased risk for schizophrenia (SCZ). The vast majority of these variants reside within non-coding sequences of the genome and are predicted to exert their effects by affecting the mechanism of action of *cis* regulatory elements (CREs), such as promoters and enhancers. Although a number of large-scale collaborative efforts (e.g. ENCODE) have achieved a comprehensive mapping of CREs in human cell lines or tissue homogenates, it is becoming increasingly evident that many risk-associated variants are enriched for expression Quantitative Trait Loci (eQTLs) and CREs in specific tissues or cells. As such, data derived from previous research endeavors may not capture fully cell-type and/or region specific changes associated with brain diseases. Coupling recent technological advances in genomics with cell-type specific methodologies, we are presented with an unprecedented opportunity to better understand the genetics of normal brain development and function and, in turn, the molecular basis of neuropsychiatric disorders. In this review, we will outline ongoing efforts towards this goal and will discuss approaches with the potential to shed light on the mechanism(s) of action of cell-type specific *cis* regulatory elements and their putative roles in disease, with particular emphasis on understanding the manner in which the epigenome and CREs influence the etiology of SCZ.

Correspondence: Dr. Panos Roussos, Icahn School of Medicine at Mount Sinai, Department of Psychiatry and Department of Genetics and Genomic Science and Institute for Multiscale Biology, One Gustave L. Levy Place, New York, NY, 10029, USA, Panagiotis.roussos@mssm.edu.

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Keywords

Postmortem; brain; gene expression; chromatin; epigenetics

Introduction

Recent years have witnessed renewed interest in studying genetic risk for SCZ, largely driven by advances in genomic technologies and a massive increase in sample sizes through the efforts of large consortia. The largest genome-wide association study (GWAS) analysis, conducted by the Psychiatric Genomics Consortium-Schizophrenia Workgroup (PGC-SCZ), comprises a sample set of 36,989 cases and 113,075 controls and identified 108 common variants that show statistical associations with SCZ (PGC-SCZ, 2014). Concurrently, the advent of next generation sequencing technologies has identified rare and *de novo* mutations conferring a high risk for the disease (Fromer et al., 2014; Purcell et al., 2014). In these exome sequencing studies, rare variants and *de novo* alleles were spread across a large number of SCZ genes, converging onto common, albeit broad, biological pathways, including genes involved in postsynaptic protein complexes and calcium signaling pathways.

Despite these efforts, a precise variant or target gene for SCZ has not been identified. There are several explanations for this, including unidentified rare variants with high penetrance or somatic mosaicism, and current methodological advances will be able to test these hypotheses in future studies. Here we focus on findings that emerge from the largest and more recent GWAS in SCZ that set out to identify common risk loci. First, the variants associated with SCZ have small effect sizes that confer moderate risk but that, collectively, contribute to SCZ (i.e. SCZ is a polygenic disease with no single variant accounting for the entire risk). We will, therefore, need to adapt current methods to allow for multiple causal variants and genes to be studied simultaneously. Second, the variants most associated with SCZ often fall within large regions of high linkage disequilibrium (LD) containing multiple variants, any of which may be driving the association. As such, additional information is required to determine which variants are more likely to have functional effects. Third, and, from the perspective of this review, perhaps most importantly, the majority of identified variants are located outside of exons and, as such, do not change the protein coding sequence of genes, suggesting a substantial role for regulatory neuroepigenomic variation in the pathogenesis of SCZ.

In this review, we first describe the neuroepigenome and our current understanding of the ways in which it can be modified. We will then discuss its role in development, how it changes across the lifespan and its impact on disease. Finally, we provide a perspective for ongoing and future approaches to further our understanding of the neuroepigenome with an emphasis on applications utilizing frozen human postmortem brain tissue. While numerous epigenomic studies have focused on peripheral tissues and animal models, the aim of this review is to discuss studies that pertain to the human brain and, more specifically, to the neuroepigenome.

What is the neuroepigenome & why is it important?

Nuclei are between 2 and 10μM in diameter yet contain approximately 2 meters of DNA. In order to fit inside the nucleus, chromosomes are packaged in to a condensed mass consisting of genomic DNA and protein, termed chromatin. Chromatin falls into two broad categories: the more densely packed, transcriptionally repressed, heterochromatin and the less densely packed, transcriptionally active, euchromatin. The basic unit of chromatin is the nucleosome, which is composed of ~147 base pairs of genomic DNA wrapped in sequence around an octamer made up of the core histone proteins, H2A, H2B, H3 and H4. Chromatin consists of arrays of nucleosomes, connected by linker DNA and linker histones, such as histone H1. The combination of histones and DNA constitute the primary building blocks of the epigenome, which comprises a regulatory network that modulates chromatin structure and, ultimately, the accessibility of specific DNA sequences to other factors, such as the molecular machinery involved in transcription. The neuroepigenome refers, specifically, to the epigenetic mechanisms (including those that modify chromatin) that contribute to brain development and function.

Importantly, the epigenome is not static and can be modified, providing a temporal dimension to gene expression and, ultimately, to cell function. Histones can undergo an array of post-translational modifications, including, but not limited to, mono-, di- and tri-methylation, acetylation and serine phosphorylation and these modifications can have a variety of impacts on genome structure and function. For example: Histone H3 methylation at lysines 4, 9, and 27, are marks associated, respectively, with active transcription, heterochromatin formation, and transcriptional repression (Li and Reinberg, 2011). Histone H3 trimethylation at lysines 27 and 9 are associated with polycomb repression and heterochromatin silencing, respectively, whereas acetylation at either residue is a characteristic of active enhancers and regulatory sequences (Ernst et al., 2011; Pasini et al., 2010; Zhu et al., 2013). Together, these provide illustrative examples of how the same residue can have a diametrically opposed influence on gene expression depending on how it is modified. For review of the different histone modifications and their impacts see (Jakovcevski and Akbarian, 2012) and references therein.

DNA sequence can also be chemically modified, leading to a variety of effects on the activity of a given gene. An example is DNA methylation, which typically results in the suppression of gene expression e.g. methylation of CpG dinucleotide islands, which are usually found in proximity to (or within) promoters. Although some genes become hypermethylated over time, there is a trend towards global loss of DNA methylation (hypomethylation) throughout life (Gonzalo, 2010), a trend that may be a contributory factor in age related neurodegenerative disorders (Akbarian et al., 2013; Johnson et al., 2012). The importance of DNA methylation in the regulation of gene expression is further demonstrated by the fact that hypermethylation and hypomethylation, relative to normal tissue, have been implicated in a variety of human cancers where, typically, there is hypermethylation of tumor suppressor genes and hypomethylation of oncogenes (Gokul and Khosla, 2013).

In addition to methylation, cytosine residues in DNA are also susceptible to modification through hydroxymethylation, in which hydrogen 5 of cytosine is replaced by a

hydroxymethyl group (5hmC). Whereas methylation occurs in promoters and is associated with lower gene expression, 5hmC, conversely, affects intragenic regions and, although it's precise role is unknown, is associated with elevated gene expression (Kato and Iwamoto, 2014; Nestor et al., 2012). During early postnatal development the neuronal genome accumulates uniquely high levels of non-CpG methylation and 5hmC (Kinde et al., 2015). Whole genome analysis has revealed that the content of 5hmC is particularly high in the brain, where it constitutes the primary modification of many enhancers and regions actively undergoing transcription (Wen et al., 2014). In addition, 5hmC peaks are found at the 5′ splice sites of exon-intron boundaries where it is thought to influence splicing and gene expression (Khare et al., 2012; Wen et al., 2014). Due to the presence of high levels of 5hmC in the brain, and in neurons, hydroxymethylation has been speculated to play a pivotal role in controlling neuronal differentiation, neural plasticity and brain functions (Wen and Tang, 2014). Genomic DNA from mouse adult brain contains high levels of 5-methylcytosine (5mC) in a non-CG context compared with other tissues (Xie et al., 2012). High levels of 5hmC have also been observed in humans, where the content of 5hmC between normal tissues appears to be highly variable, is associated with the body of transcribed genes, and is directly proportional to levels of transcription of those genes (Nestor et al., 2012).

Epigenetic modification of DNA has also been identified as a key mechanism for environmental regulation of gene expression (Jirtle and Skinner, 2007) and environmental factors can trigger lifelong molecular changes to the epigenome with a profound impact on the health and, perhaps, behavior of the organism later in life (Klengel and Binder, 2015). Although the majority of epigenetic research has focused on modifications of histones and DNA, RNA is also extensively modified (Satterlee et al., 2014). RNA methylation has been observed in both prokaryotic and eukaryotic organisms and in numerous types of RNA molecules, including mRNA, tRNA, and non-coding RNA (Wang and He, 2014). Although the function of RNA methylation remains unclear, it has been proposed to play roles in, among others, post transcriptional regulation of gene expression (Yue et al., 2015) and RNA biogenesis and splicing (Alarcon et al., 2015a; Alarcon et al., 2015b; Dominissini et al., 2012).

The genome in 3-Dimensions

Importantly, DNA methylation and its variants (hydroxymethylation, etc.), multiple post-translational histone modifications and other types of epigenetic regulation, fail to fully characterize the epigenome and localized chromatin architecture at any given genomic locus. This is because the chromosomal arrangements in the interphase nucleus are not random and it is now generally accepted that genetic information is not only encoded in nucleotide sequence but also in the dynamic 3-dimensional organization of the genome. For example, loci at sites of active gene expression are more likely to be clustered together and positioned towards a central position within the nucleus, while heterochromatin and silenced loci are located towards the nuclear periphery (Cremer and Cremer, 2001; Duan et al., 2010). Thus, the spatial position of genomic sequences provides a critically important layer of regulation in eukaryotic cells. Furthermore, chromosomal loopings are associated with transcriptional regulation by permitting direct interaction between distal DNA elements, often separated by

many kilobases along the linear genome (Gaszner and Felsenfeld, 2006; Sanyal et al., 2011; Wood et al., 2010).

Some interactions influence fundamental biological processes such as imprinting (Zhang et al., 2014) and dysregulated higher order chromatin is also thought to contribute to disease, for example Cornelia de Lange Syndrome (CdLS). With an estimated incidence of 1:10–30,000 live births, CdLS is among the more frequent genetic disorders (source http://ghr.nlm.nih.gov). CdLS is associated with a range of neuropsychiatric symptoms, including various manifestations of psychosis (Moss et al., 2008), and is likely to be caused by mutations in the cohesin complex (Deardorff et al., 2012; Gervasini et al., 2013), which functions to promote the physical interaction of distal promoters and enhancers to regulate gene expression (Kagey et al., 2010).

The full extent to which the 3-D structure of chromatin influences cell function is unclear and awaits further investigation using novel technologies (Cattoni et al., 2015). Over the last decade, several approaches have been developed to determine the frequency with which any two loci in the genome are in close enough physical proximity to interact. All of these approaches are based on Chromosome Conformation Capture (3C) (Dekker et al., 2002). Using 3C and its iterations, thousands of significant long-range interactions between gene promoters and distal loci have been identified (Sanyal et al., 2012). Importantly, ligation-based chromosome conformation capture is a technique that is applicable to postmortem brain (Mitchell et al., 2014b).

The neuroepigenome in development

Unlike the underlying genome, the composition of the epigenome can be dynamically modified during development and both histones and DNA can display the hallmarks of epigenetic modification (Zhou et al., 2011a). The epigenome is, therefore, variable, and this variability is a major determinant of the distinct patterns of gene expression observed across a wide array of developmental stages, cell lineages, and environmental conditions (Bernstein et al., 2007). Lister and colleagues examined both mouse and human frontal cortex during a broad range of developmental stages and demonstrated that widespread reconfiguration of the methylome occurs during fetal to young adult development. During this period (when synaptogenesis and synaptic pruning are at their peak), highly conserved non-CG methylation (mCH) accumulates in neurons, but not glia, to become the dominant form of methylation in human neurons (Lister et al., 2013). More recently, genome-wide analysis examined DNA methylation in human fetal brain samples, ranging from 23 to 184 days post-conception (Spiers et al., 2015). Significant changes in DNA methylation during brain development were observed, characterized by an enrichment of hypomethylated loci with fetal age. Moreover, during early postnatal development, high levels of non-CpG methylation and hydroxymethylation accumulate in neurons (Gabel et al., 2015; Kinde et al., 2015). Furthermore, Shulha and co-workers examined a histone modification marker of promoters - H3K4 trimethylation (H3K4me3) - in chromatin isolated from neurons of subjects across a developmental spectrum ranging from late in gestation to 80 years of age. Their work revealed that histone methylation is highly dynamic during the late prenatal

period and the first year after birth, and that this trend is maintained throughout life, albeit with increasingly slower kinetics over time (Shulha et al., 2013).

Zhu and co-workers examined multiple epigenomic marks in twenty-nine different tissue and cell types, including six distinct brain regions (anterior caudate, cingulate gyrus, hippocampus, middle inferior temporal lobe, mid-frontal lobe and substantia nigra) (Zhu et al., 2013). Assays included analysis of H3K4methylation and H3K9 and H3K27 methylation and acetylation. Histone modifications associated with diverse regulatory and epigenetic functions across different developmental stages, lineages, and cellular environments were identified, emphasizing the need to carry out cell- and tissue-specific analysis in pursuit of a more comprehensive understanding of the mechanisms underlying development, normal cellular function and disease. Interestingly, neurons have been shown to display a distinctive DNA methylation profile with greater inter-individual variation when compared with non-neurons (Iwamoto et al., 2011).

The role of the neuroepigenome in schizophrenia

Numerous studies link dysregulation of the epigenome to disease [for examples see (Robertson, 2005) (Mirabella et al., 2015)] such as cancer (Deb et al., 2014), heart disease (Zhang and Liu, 2015) and metabolic disorders (Martinez-Jimenez and Sandoval, 2015). In addition, a number of groups have attempted to assess the role of the epigenome in the etiology of neurological disorders, including autism (Shulha et al., 2012a), addiction (Zhou et al., 2011b), Huntington's disease (HD) (Bai et al., 2015), multiple sclerosis (MS) (Huynh et al., 2014) and Alzheimer's disease (De Jager et al., 2014) (Lunnon et al., 2014).

Over the past decade an increasing number of studies have also investigated a role for the epigenome in the etiology of SCZ, with initial work focusing on the impact of differential methylation on the expression of specific genes, such as Reelin (*RELN*) (Grayson et al., 2005) (Abdolmaleky et al., 2005), membrane-bound catechol-O-methyltransferase (*COMT*) (Abdolmaleky et al., 2006), Sex Determining Region Y-Box 10 (*SOX10*) (Iwamoto et al., 2005), serotonin receptor 2A (*HTR2A*) (Abdolmaleky et al., 2011) and 1A (*HTR1A*) (Carrard et al., 2011), brain-derived neurotrophic factor (*BDNF*) and the dopamine transporter (*DAT1*) (Kordi-Tamandani et al., 2012) (Table 1).

With the advent of new, cost effective, genome-wide approaches, more recent endeavors have attempted to assess links between the neuroepigenome and disease in an unbiased, global, manner. By examining 12,000 CpG islands in the frontal cortex of SCZ cases (n=35), Mill and colleagues revealed differential DNA methylation in genes associated with glutamatergic and GABAergic pathways when compared to controls (n=35) (Mill et al., 2008). DNA methyltransferase1 (*DNMT1*) and ten-eleven translocase methylcytosine dioxygenase1 (*TET1*) belong to families of enzymes that methylate and hydroxymethylate, respectively, CpG islands of many gene promoters. Overexpression of *DNMT1* and *TET1* was observed in the brain of SCZ and Bipolar (BP) patients coupled with increased binding of *DNMT1* to a subset of GABAergic (e.g. *GAD1*) and glutamatergic (e.g. BDNF-IX) promoters (Dong et al., 2014). The *GAD1* regulatory network plays a role in chromatin regulation and cell cycle control and, more recently, hypermethylation of components of the

GAD1 family has been implicated in SCZ and BP (Ruzicka et al., 2015). Interestingly, the increased binding of *DNMT1* to GABAergic and glutamatergic promoters was detected in the cerebral cortex but not in the cerebellum, suggesting a brain region specific mechanism and further emphasizing the need to carry out cell- and tissue-specific analysis when studying disorders of the brain (Dong et al., 2014). For further information on the role of *GAD1* in SCZ see (Mitchell et al., 2014c).

Pidsley and colleagues assessed levels of DNA methylation in two brain regions (prefrontal cortex and cerebellum) from SCZ patients and controls (Pidsley et al., 2014). Differentially methylated loci were identified in regions of the genome rich in genes implicated in neurodevelopment. In addition, genome-wide methylation and gene expression profiles of brain tissue (cerebellum) from controls (n=43), BP patients (n=36) and patients with SCZ (n=39) identified a strong correlation between gene expression and methylation for a number of genes, including nescient helix-loop-helix 1 (*NHLH1*) (a.k.a. NSCL1) (Chen et al., 2014a) a gene expressed during neurodevelopment (Lipkowitz et al., 1992). Genome-wide analysis of CpG methylation sites (27,578 CpGs spanning 14,495 genes) of human postmortem tissue isolated from the dorsolateral prefrontal cortex (DLPFC) of healthy controls and individuals with SCZ, identified aberrant DNA methylation at 107 CpG sites associated with SCZ. Of these sites, 73.8% were hypermethylated. Furthermore, a large number of *cis*-methylation quantitative trait loci (mQTL) were identified, including associations with known SCZ risk variants (Numata et al., 2014). Similarly, Wockner and colleagues compared DNA methylation of the frontal cortex between cases with SCZ (n=24) and controls (n=24) (Wockner et al., 2014). 2929 differentially methylated genes were found, of which 1291 (44%) were located in CpG islands and 817 (17.1%) in promoter regions. More than 100 of these genes overlap with a previous DNA methylation study of peripheral blood from SCZ patients in which 27,000 CpG sites were analyzed (Nishioka et al., 2013) demonstrating that, while analysis of peripheral cells can be informative, directly studying cells of the brain is likely to provide a more thorough understanding of brain function. Indeed, early analysis has provided evidence that SCZ single nucleotide polymorphisms (SNPs) might have a pleotropic effect on the epigenomic regulation of gene expression in other tissues besides the brain (PGC-SCZ, 2014), however, the enrichment is expected to be lower in tissues other than the organ of interest (i.e. brain). Similar to SCZ, widespread changes in DNA methylation have also been observed in BP. Genome-wide DNA methylation profiling using methylated DNA immunoprecipitation followed by sequencing (MeDIP-seq) uncovered differentially methylated regions (DMRs) in two brain regions (frontal cortex and anterior cingulate) of SCZ (n=5) and BP (n=7) relative to controls (n=6). In SCZ and BP, the different brain regions display distinct patterns of methylation, with hypomethylation observed in the frontal cortex while the anterior cingulate displayed extensive hypermethylation (Xiao et al., 2014). Interestingly, DMRs in the same brain regions from SCZ and BP could successfully distinguish BP and/or SCZ from normal controls while differentially expressed genes could not (Xiao et al., 2014). Aberrant modification of histones has also been implicated in neuropsychiatric disorders. H3K9me2 is associated with a restrictive chromatin state, leading to reduced levels of transcription. Chase and colleagues demonstrated, by immunoblotting, an increase of H3K9me2 in the parietal cortex of cases with SCZ compared to controls and showed that

increased histone methyltransferase (HMT) mRNA transcript levels correlated with the severity and duration of symptoms of SCZ, as well as family history (Chase et al., 2013).

In addition to methylation etc., alterations in chromosomal loop structures have also been implicated in disease, having been shown to affect expression of GABA synthesis genes (Bharadwaj et al., 2013), NMDA glutamate receptor subunits (Bharadwaj et al., 2014) and the calcium channel gene *CACNA1C* (Roussos et al., 2014) in the prefrontal cortex of subjects with SCZ. Long-range intrachromosomal interactions, therefore, offer an additional explanation for the initially surprising finding that many SCZ risk loci reside within non-coding sequences.

Taken together, the data supports a major role for brain-region and cell-type-specific epigenomic differences in the pathogenesis of neuropsychiatric disorders, including SCZ. It is also evident that tissue and cell-type specific assays are essential if a thorough understanding of the underlying genetic causes of SCZ is ever to be achieved. The majority of studies to date have employed microarrays to examine CpG methylation. In order to generate a more complete picture of the role of the epigenome in normal development and disease, the study of other epigenetic modifications using newer technologies that assess the neuroepigenome, genome-wide, is required (Maunakea et al., 2010). This strategy holds the promise to elucidate the function of non-coding, disease-associated loci, and moves towards the development of testable hypotheses regarding biological processes involved in the pathogenesis of SCZ and other disorders. For further information see the following reviews and the references therein: (Keverne, 2014) (Svrakic et al., 2013) (Gavin and Floreani, 2014) (Akbarian, 2014) (Jakovcevski and Akbarian, 2012).

As is the case with most studies of SCZ the potential influence of treatment with antipsychotic drugs cannot/should not be overlooked. A case in point is a recent study by Melka et al. (2014) that showed increased (~1,200 genes) and decreased (~550 genes) methylation of genes in multiple brain regions of rats treated chronically with therapeutic relevant doses of the antipsychotic drug olanzapine (Melka et al., 2014).

Recent large-scale efforts to study the non-coding genome

Large-scale, coordinated, efforts are required to systematically explore the regulatory function of the non-coding genome. Projects such as ENCyclopedia Of DNA Elements (ENCODE) project (ENCODE Project Consortium, 2012) (Bernstein et al., 2012; Maurano et al., 2012), the NIH Roadmap Epigenome Mapping Consortium (REMC) (Bernstein et al., 2010; Roadmap Epigenomics et al., 2015) and FANTOM5 (Andersson et al., 2014) have made great strides towards providing a detailed catalogue of CREs in the human genome. As in other common diseases, SCZ-associated variants are enriched in these functional non-coding elements (Roussos et al., 2014). In particular, genetic variants associated with SCZ overlap CRE subgroup annotations in specific cell types in the brain that define active enhancers and promoters (e.g. histone modification H3K4me3), and within the broad functional class of deoxyribonuclease I (DNase I) hypersensitive sites (DHSs) (Gusev et al., 2014). Importantly, the enrichments are specific for cell types related to the disease (Maurano et al., 2012; Trynka et al., 2013). Therefore, integrating functional information

with genetic association data has been shown to be a useful tool to improve fine-mapping accuracy, as well as to provide insights into the underlying biological mechanisms by identifying relevant tissue-specific functional elements (Gusev et al., 2014; Kichaev et al., 2014; Pickrell, 2014).

However, application of existing epigenome data is challenging and limited for several reasons. First, ENCODE (Bernstein et al., 2012; Maurano et al., 2012) focused primarily on various actively dividing cell lines and tissues: no brain tissue specimens were included. Thus, findings from the ENCODE project will require follow-up work utilizing human brain tissue. Second, REMC (Roadmap Epigenomics et al., 2015; Zhu et al., 2013) assessed multiple chromatin modification markers, including assays for promoters and enhancers and, third, FANTOM5 generated annotations for enhancers using cap analysis gene expression. Both REMC and FANTOM5 employed assays using cell-type nonspecific brain tissue homogenates from a small number (N<3) of control brains only. Within brain tissue, however, neurons are intermingled with, and outnumbered by, multiple types of non-neuronal cells (microglia, oligodendrocytes and astrocytes). This is important, since SCZ-associated abnormalities have been demonstrated in specific cellular populations, including neocortical neurons (Benes et al., 2001; Chen et al., 2014b; Rajkowska et al., 2001), astrocytes (McCullumsmith et al., 2015; Schnieder and Dwork, 2011), oligodendrocytes (Haroutunian et al., 2014; Mighdoll et al., 2015; Roussos and Haroutunian, 2014) and microglia (Bernstein et al., 2015). Furthermore, CRE-mediated epigenetic regulation shows cell type specificity (Cheung et al., 2010; Heintzman et al., 2009; Maurano et al., 2012; Roadmap Epigenomics et al., 2015). Therefore, the study of mixed cell populations can mask cell-type specific signals. In addition to cell-type specific annotations, inclusion of functional data from large sample sized sets of controls and cases will provide sufficient statistical power to identify robust differences in CREs altered in psychiatric disorders, as well as measuring the effect of genetic variants on the *cis* regulation of gene expression.

Ongoing efforts from large brain-focused consortia, including the CommonMind Consortium and PsychENCODE aim to generate detailed region- and cell-type specific annotation maps of the transcriptome and epigenome, and to identify alterations associated with neuropsychiatric diseases. The goal of the CommonMind Consortium (www.synapse.org/cmc) is to generate and analyze large-scale transcriptome data from brain specimens of controls subjects and cases with SCZ and BP. The PsychENCODE consortium was recently established to study the epigenome landscape of neuropsychiatric diseases (Akbarian et al., 2015). The project focuses on 3 neuropsychiatric diseases (autism spectrum disorder, BP and SCZ). PsychENCODE aims to characterize the epigenome landscape of the brain in a large cohort of cases and controls, as well as to functionally characterize disease-associated regulatory variants in model systems. Completion of large projects focusing on the neuroepigenome will shed light onto the regulatory mechanisms of SCZ-associated variants.

Future approaches to better characterize the non-coding regulatory regions of the genome

In the following section we outline ongoing and future approaches to analyze dynamic modifications of chromatin and further our understanding of the structure and function of non-coding regulatory regions of the genome. Ideally, such approaches will facilitate the study of the neuroepigenome at a cell-type specific resolution while utilizing low amounts of input material.

Cell-type specific analysis within the postmortem brain

In cortical gray matter, significant differences are observed when comparing the distribution of trimethylated histone H3K4 (H3K4me3) in neurons (identified using an antibody against the neuron-specific antigen, NeuN) to that of non-neurons (NeuN-) in cell populations residing in the same tissue, the prefontal cortex (PFC) (Cheung et al., 2010; Shulha et al., 2012b). Therefore, the study of homogenous cell populations may fail to distinguish signals unique to specific cell-types, potentially missing critical changes in discrete cell populations. To overcome these limitations it is, therefore, necessary to conduct cell-type specific epigenome studies. Working with frozen postmortem tissue presents unique challenges, however, including loss of cytoplasm (and, with it, many cell-specific antigens) as a consequence of freeze-thawing, compromise of tissue integrity due to variable postmortem interval (PMI) and diverse agonal and pre-agonal events, impacting the quality of biological material. The isolation of cell-specific nuclei is hampered by a limited repertoire of available antibodies against cell-type specific nuclear antigens. Despite this, Jiang and colleagues developed a fluorescence-activated cell sorting (FACS)-based method for separating neuronal and non-neuronal nuclei from frozen brain samples using the anti-NeuN antibody (Jiang et al., 2008). Similar cell-type specific nuclear antigens are increasingly being utilized to isolate other brain cell populations (for example glutamatergic and GABAergic neurons (Kozlenkov et al., 2015)), ultimately allowing for the generation of comprehensive region and cell-specific maps of the neuroepigenome from frozen postmortem brain tissue. Coupled with advances in genomics and increased data generation from small numbers of cells, conducting cell-type specific analysis of the epigenome with limited amounts of human brain tissue is becoming increasingly achievable.

Whole-genome bisulfite sequencing

DNA methylation is critically important to regulate gene expression and cellular functions (Bibikova and Fan, 2010). So far, various techniques have been developed to profile DNA methylation but most do not allow for measuring methylation status in large sample sets at high resolution and may be insensitive to subtle, disease associated, methylation changes. By treating DNA with bisulfite it is possible to introduce specific DNA sequence changes based on the methylation status of individual cytosine residues. The development of genome-wide DNA methylation profiling technologies has made it possible to interrogate DNA methylation status over large genomic regions. Lister et al. compiled the first genome-wide, single-base resolution, methylation map using whole-genome bisulfite sequencing (WBBS) covering more than 90% of the approximately 28.7 million CpGs in the human

genome (Lister et al., 2009). However, this accuracy and resolution demands much higher sequencing reads which is, for the time being, expensive.

Chromatin immunoprecipitation followed by sequencing

Chromatin immunoprecipitation (ChIP) is a method to identify fragments of genomic DNA bound by a particular protein. ChIP works by enriching specific crosslinked DNA-protein complexes using an antibody against the protein of interest, such as a transcription factor (Mahony and Pugh, 2015). Provided the appropriate antibody exists, ChIP can also be employed to isolate DNA bound by modified proteins (e.g. methylated or acetylated histones). ChIP-seq combines DNA fragment isolation by ChIP with next-generation sequencing in order to measure the genome-wide distribution of DNA binding proteins. Numerous studies have employed ChIP-seq approaches to further our understanding of transcription, the epigenome and the neuroepigenome (Maze et al., 2014). Traditionally, ChIP-seq protocols have required relatively large amounts of sample (~10 million cells), limiting their application to the study of readily available tissue (Huang et al., 2006) (Furey, 2012). In addition, these approaches have traditionally employed tissue homogenates, thereby failing, by necessity, to address the cellular diversity found in the brain (Mitchell et al., 2014a). However, an increasing number of new methods have become available that are compatible with low-input (Adli and Bernstein, 2011; Brind'Amour et al., 2015; Jakobsen et al., 2015; Lara-Astiaso et al., 2014; Ng et al., 2013; Schmidl et al., 2015; Shankaranarayanan et al., 2011; Zwart et al., 2013). Coupled with FACS, these approaches facilitate the application of ChIP-seq to the study of low abundance cell-types in relatively small amounts of tissue.

Open chromatin assays to identify potential *cis* regulatory elements

The nucleosome is known to play a central role in regulating gene transcription from promoters and exists in a dynamic equilibrium between open and closed states (Mellor, 2005). Nucleosome rearrangement (leading to open chromatin) at promoters and enhancers results from the binding of specific regulatory factors responsible for transcriptional activation (Henikoff, 2008). Open or accessible regions of the genome are regarded as primary positions for regulatory elements and are crucial in mediating gene expression (John et al., 2011). Approaches such as DNase-seq (DNaseI hypersensitivity regions) (Song et al., 2011) or FAIRE-seq (Formaldehyde Assisted Isolation of Regulatory Elements) (Simon et al., 2012), have been utilized to map open chromatin (Maurano et al., 2012), however, these techniques are limited in that they require large amounts of input material, thereby prohibiting cell type specific studies where source material is limiting. More recently, a tagmentation based method called Assay for Transposase Accessible Chromatin followed by Sequencing (ATAC-seq) has been developed (Buenrostro et al., 2013). ATAC-seq employs a transpososome complex to insert oligonucleotides into regions of the genome that are sufficiently open to facilitate its entry. The oligonucleotides then provide a means of generating, via polymerase chain reaction (PCR), sequencing libraries enriched for open chromatin with sufficient resolution to map transcription factor occupancy and nucleosome positions in regulatory sites. ATACseq offers substantial advantages over DNase-seq or

FAIRE-seq due to its speed, simplicity, and low input cell number requirement (~50,000 cells).

Refining the search to identify active enhancer elements

All of the aforementioned approaches allow for the genome wide identification of potential regulatory elements, however, they fail to provide a direct functional readout of the activity of these elements. In order to fully understand the genetics underpinning brain development and function in health and disease, it will be necessary to distil down this broad inventory of putative regulatory elements to a list of the most salient actors. Several different methodologies render this goal possible. Techniques such as STARR-seq (Self-Transcribing Active Regulatory Region sequencing) (Arnold et al., 2013) and FIREWACh (Functional Identification of Regulatory Elements Within Accessible Chromatin) (Murtha et al., 2014), allow for the identification of active cell-type specific regulatory elements from libraries of fragmented, or pre-selected, DNA, enabling the rapid screening of entire genomes, reviewed in (Dailey, 2015). STARR-seq and FIREWACh take advantage of the observation that enhancers can work independent of their relative locations and both methods use reporter assays to interrogate DNA populations for elements capable of driving transcription. By directly coupling candidate sequences to enhancer activity these approaches enable the evaluation of millions of DNA fragments in a single experiment. A critical consideration with each approach, however, is the relevance of the cells used to carry out the assay and the transcriptional programs therein.

Linking the activity of enhancers to specific genes

Having identified active *cis* regulatory elements within a given cellular context, the next requirement would be to assign their activities to a specific gene, or set of genes. Chromosome conformation capture (3C) based methodologies are a useful tool towards this purpose, as they allow for the identification of physical interactions between distal genetic elements, e.g. between a gene and an enhancer (Naumova et al., 2012; Simonis et al., 2007), and can range from target-specific (3C) to unbiased, genome-wide, approaches (Hi-C) (Dekker et al., 2013). 3C involves cross-linking of interacting DNA segments, followed by digestion with a frequently cutting restriction enzyme. Digested DNA is then re-ligated, cross-links are reversed and the resulting 3C library subjected to PCR using primers that flank putative ligation junctions, thereby assessing the frequency at which otherwise distal genetic elements ligate to one another; a reflection of their physical proximity within chromatin. An increasing number of studies have applied 3-C based techniques to the study of neuropsychiatric disorders (Bharadwaj et al., 2014; Roussos et al., 2014) and they provide a direct means of identifying functional relationships between genes and *cis* regulatory elements.

The future of genome wide analysis of precious materials – less is more

A major limitation of traditional epigenomic approaches (e.g. ChIPseq) is the large number of cells required to generate high-quality data sets. The ATACseq method has recently been modified to allow profiling of chromatin accessibility at the resolution of single cells

(Buenrostro et al., 2015; Cusanovich et al., 2015). A number of new approaches now allow for the performance of ChIP-seq, genome wide transcription factor binding and methylome analysis using as little as 1000 cells (Adli and Bernstein, 2011; Adli et al., 2010; Brind'Amour et al., 2015; Schmidl et al., 2015). Furthermore, additional tagmentation-based methodologies have been developed to facilitate analysis of the complete methylome by whole-genome bisulfite sequencing, requiring as little as 10ng of input DNA (~3000 cells) (Adey and Shendure, 2012; Lipka et al., 2014; Wang et al., 2013). For more information on low input approaches see (Greenleaf, 2015).

Although a number of tissue repositories exist worldwide, well-characterized and clinically relevant material is a critical and very precious resource. It is, therefore, imperative to utilize approaches that can minimize input while maximizing informational yield. All of these low-input approaches, when combined with chromatin-state capture methods and next generation sequencing, portend the near-future prospects of understanding the neuroepigenome, not only with cell-type specificity, but in the context of anatomically discrete brain regions and nuclei. These improved methods will not only increase our knowledge of the normal functions of the brain during the lifespan of an individual, but will provide a deep and functional genomic understanding of the neurobiology of brain diseases, including schizophrenia and bipolar disorders.

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Table 1

Description of neuroepigenome studies in neuropsychiatric diseases.

Type	Target	Tissue/cells	Disease	Assay	Main Findings	Reference
Targeted Analysis	reelin (*RELN*)	occipital cortex	SCZ	Bisulfite DNA sequencing	Promoter hypermethylation leads to reduced expression of reelin in SCZ	(Grayson et al., 2005)
		frontal lobe	SCZ	Methylation specific PCR		(Abdolmaleky et al., 2005)
	Sex Determining Region Y-Box 10 (*SOX10*)	prefrontal cortex	SCZ	Bisulfite DNA sequencing	CpG island hypermethylation correlated with reduced expression of SOX10 in SCZ	(Iwamoto et al., 2005)
	catechol-O-methyltransferase (*COMT*)	frontal lobe	SCZ BP	Methylation specific PCR	Hypomethylated MB-COMT promoter in SCZ and BP leads to increase in MB-COMT transcript levels	(Abdolmaleky et al., 2006)
	serotonin receptor 2A (HTR2A)	frontal lobe	SCZ	Bisulfite DNA sequencing	HTR2A promoter was hypermethylated at and around the -1438A/G polymorphic site, but hypomethylated at and around T102C polymorphic site in SCZ and BD. Down-regulation of HTR2A associated with early age of disease onset in SCZ and BD	(Abdolmaleky et al., 2011)
	serotonin receptor 1A (*HTR1A*)	blood leukocytes	SCZ BP	Methylation via high resolutoin melt (HRM)	Promoter hypermethylation of the 5HTR1A gene in SCZ and BD	(Carrard et al., 2011)
	brain-derived neurotrophic factor (*BDNF*)	peripheral blood	SCZ	Methylation specific PCR	Decreased methylation frequency of the BDNF gene in SCZ with corresponding increase in gene expression	(Kordi-Tamandani et al., 2012)
	GABA synthesis genes	prefrontal cortex	SCZ	histone H3K4 trimethylation Chromosome conformation capture (3C)	Chromosomal loopings for promoter-enhancer interactions involved in the regulation of GABAergic gene expression are disordered in some cases with SCZ	(Bharadwaj et al., 2013),
	CACNA1C	prefrontal cortex	SCZ	3C	Establishes a functional link between noncoding SNPs and 3D genome architecture in SCZ	(Roussos et al., 2014)
Genome-wide Analysis	Genomics	frontal cortex	SCZ BP	CpG island microarrays	Differential DNA methylation in genes	(Mill et al., 2008).

Type	Target	Tissue/cells	Disease	Assay	Main Findings	Reference
					associated with glutamatergic and GABAergic pathways	
		prefrontal cortex	SCZ	Genome wide DNA methylation profiling	Aberrant DNA methylation in schizophrenia was identified at multiple CpG sites	(Numata et al., 2014).
		frontal cortex	SCZ	Genome wide DNA methylation profiling	2929 unique genes were found to be differentially methylated, including the SCZ associated genes NOS1, AKT1, DTNBP1, DNMT1, PPP3CC and SOX10	(Wockner et al., 2014)
		frontal cortex, anterior cingulate	SCZ BP	methylated DNA immunoprecipitation followed by sequencing (MeDIP-seq)	Hypomethylation observed in the frontal cortex while the anterior cingulate displayed extensive hypermethylation in both SCZ and BP	(Xiao et al., 2014).
		prefrontal cortex, cerebellum	SCZ	Genome wide DNA methylation profiling	Differential DNA methylation at multiple loci wirth enrichment for genes involved in neurodevelopmental processes	(Pidsley et al., 2014)
		cerebellum	SCZ BP	Genome wide DNA methylation profiling	differentially expressed genes with an aberrant methylation pattern associated with SCZ and PB, including PIK3R1, BTN3A3, NHLH1 and SLC16A7	(Chen et al., 2014a)
		hippocampus	SCZ BP	Bisulfite-pyrosequencing	Hypermethylation of components of the GAD1 family in SCZ and BP	(Ruzicka et al., 2015).
		peripheral blood	SCZ	Genome wide DNA methylation profiling	Aberrant methylation of DNA has been associated with the discordance for SCZ between monozygotic twins	(Castellani et al., 2015)
		entorhinal cortex, superior temporal gyrus, prefrontal cortex, cerebellum	AD	DNA methylation array Bisulfite-pyrosequencing	Cortex-specific hypermethylation of ANK1 correlates with AD-associated neuropathology	(Lunnon et al., 2014)
		prefrontal cortex	AD	DNA methylation sequencing	Methylation levels at 71 of 415,848 interrogated CpGs was significantly associated	(De Jager et al., 2014

Type	Target	Tissue/cells	Disease	Assay	Main Findings	Reference
					with AD pathology including the genes ANK1, CDH23, DIP2A, RHBDF2, RPL13, SERPINF1 and SERPINF2	
		prefrontal cortex	Autism	H3K4me3 ChIP-seq	Prefrontal cortex neurons from subjects with autism show genome-wide changes in chromatin structures	(Shulha et al., 2012a)
	Immunohisto-chemistry	prefrontal cortex	SCZ	immunohistochemical analysis, DNA arrays, and RT-PCR	High levels of H3-(methyl)arginine 17 are associated with down-regulated metabolic gene expression in SCZ	(Akbarian et al., 2005)
		temporal neocortex	AD	DNA methylation specific antibodies	significantly reduced levels of DNA methylation in temporal neocortex neuronal nuclei of the affected twin in set of monozygotic twins discordant for AD	(Mastroeni et al., 2009)
		hippocampus	AD	5-mC and 5-hmC immunochistochemistry	decrease in levels of 5-mC and 5hmC in AD	(Chouliaras et al., 2013)
		entorhinal cortex, cerebellum	AD	DNA methylation specific immunofluorescence	Decrease in global 5-hmC in entorhinal cortex and cerebellum, and differences in 5-formylcytosine levels between brain regions in AD	(Condliffe et al., 2014)

Table 2

Overview of large-scale epigenomics projects

Project	Source material	Case/Control Comparison	Methods employed	Reference
ENCODE	cell lines	N	5C, RNA-seq, CAGE, RNA-PET, ChIA-PET, ChIP-seq, CLIP-seq, DNase-seq, FAIRE-seq, RRBS, WGBS	(ENCODE Project Consortium, 2012)
REMC	stem cells, postmortem tissue, cell lines	N	WGBS, ChIP-seq, RNA-seq	(Bernstein et al., 2010)
Fantom 5	tissues, primary cells, cell lines	N	DAGE, DNase-seq, ChIP-seq (H3K4me1, H3K27ac)	(Andersson et al., 2014)
PsychENCODE	postmortem brain, iPSCs	Y	ChIP-seq (H3K4me3, H3K27ac), ATAC-seq, 3C, STARR-seq, WGBS, RNA-seq, NOME-seq, MWA-RPPA	(Akbarian et al., 2015)

3C: Chromosome conformation capture

5C: Carbon-Copy Chromosome Conformation Capture

ATAC-seq: Assay for Transposase Accessible Chromatin followed by Sequencing

CAGE: cap analysis of gene expression

ChIA-PET: Chromatin Interaction Analysis by Paired-End Tag Sequencing

ChIP-seq :chromatin immunoprecipitation followed by sequencing

CLIP-seq: cross-linking immunoprecipitation sequencing

DNase-seq: deoxyribonuclease I (DNase I) hypersensitive site sequencing

FAIRE-seq: Formaldehyde assisted identification of regulatory elements followed by sequencing

FIREWACh: Functional Identification of Regulatory Elements Within Accessible Chromatin

H3K27ac: histone 3 Lysine 27 acetylation

H3K4me1: histone 3 Lysine 4 monomethylation

H3K4me3: histone 3 Lysine 4 trimethylation

iPSCs: induced pluripotent stem cells

MWA-RPPA: micro-western arrays coupled with reverse-phase protein arrays

NOMe-seq: Nucleosome occupancy and methylome sequencing

RNA-PET: RNA Paired-End tags

RNA-seq: RNA sequencing

RRBS: Reduced representation bisulphite sequencing

STARR-seq: Self-transcribing active regulatory region sequencing

WGBS: Whole genome bisulfite sequencing