Handbook of Clinical Neurology, Vol. 150 (3rd series)
Brain Banking
I. Huitinga and M.J. Webster, Editors
https://doi.org/10.1016/B978-0-444-63639-3.00017-7

Chapter 17

Epigenetic analysis of human postmortem brain tissue

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Abstract

Epigenomic profiles have been mapped across a broad range of brain regions and developmental contexts in postmortem human brain tissues, illuminating our understanding of epigenetic regulation in neural function and plasticity across the life course. Importantly, disease-associated epigenetic alterations in postmortem brain have provided compelling insights into the gene-regulatory architecture underlying neurobiologic disease susceptibility and pathogenesis. However, the use of postmortem brain tissues for molecular analyses warrants careful consideration of key technical and biologic factors that may confound epigenetic analyses. In this chapter, we describe the predominant forms of epigenetic regulation (DNA modifications, chromatin structure, and noncoding RNA expression) and discuss the various methodologies used to assess each epigenetic mark. In addition, we provide an overview of existing epigenetic studies using human brain tissues as well as highlight the various challenges and considerations for epigenomic profiling in human postmortem brain samples.

INTRODUCTION

Over the last decade, the field of neuroepigenetics has garnered considerable interest for its potential to provide insights into the molecular mechanisms underpinning neurologic function and brain-related pathologies. While genetic variation comprises the inherited basis of brain function and activity, epigenetics is considered the regulatory overlay of the genome that modulates gene activity in response to external signals (Boyce and Kobor, 2015). The concept of epigenetic regulation was first proposed by Conrad Waddington in the early 1940s to describe how the developmental patterning of multicellular organisms is shaped by "epigenetic landscapes" that drive cellular differentiation along a programmed trajectory towards specific cell type lineages (Waddington, 1968). Since its inception, the field of epigenetics has flourished into an active area of study aimed at characterizing gene regulation and biologic variation.

Today, epigenetics is operationally defined as modifications of DNA and its regulatory components, including chromatin and noncoding RNA (ncRNA), to potentially modulate gene transcription without changing the DNA sequence itself (Bird, 2007; Meaney, 2010; Henikoff and Greally, 2016). Notably, Waddington's original hypothesis still holds true: the identity and the functional specification of the ~200 different cell types in the human body are largely dictated by the unique epigenomic profiles and corresponding transcriptional activity of each cellular subtype. In this manner, epigenetic mechanisms dually serve to allow for dynamic tissue- and cell type-specific variation, as well as the preservation of the cellular memory required for developmental stability. Importantly, epigenetic regulation serves as one of the most promising candidates for the biologic mediation of gene–environment interactions (Meaney, 2010).

This tissue-specific and environmentally responsive control of gene regulation makes epigenetics a prime

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research focus for molecular studies using human postmortem brain samples, particularly in the context of complex, environmentally-conditioned neurobiologic and psychiatric phenotypes. Specifically, analyses in postmortem brain tissues provide the unique opportunity to perform in-depth molecular characterizations of the brain that are not possible in living subjects. As such, direct examination of epigenetic profiles in target brain tissues has become a compelling theme in human health research; epigenomic studies in postmortem brain are becoming increasingly prevalent in developmental, neurologic, psychiatric, and neurodegenerative disease fields. In this chapter, we describe the predominant modes of epigenetic regulation, provide an overview of existing epigenetic studies using human brain tissues, and finally discuss the various challenges and considerations for epigenomic profiling in human postmortem brain samples.

OVERVIEW OF EPIGENETIC MECHANISMS AND METHODOLOGIES

The orchestration of epigenetic control involves a number of distinct mechanisms: (1) DNA modifications; (2) regulation of chromatin structure; and (3) ncRNA activity. Here we outline these three main types of epigenetic regulation and the different methods that can be used to measure them (Fig. 17.1).

DNA modifications

Modifications of DNA nucleotides are a long-established form of epigenetic regulation, and include both DNA methylation (DNAm) and hydroxymethylation (DNAhm). Of these, DNAm is perhaps the best-characterized epigenetic modification and refers to the covalent attachment of a methyl group to the 5' position of cytosine, generally occurring at cytosine-guanine dinucleotide (CpG) sites (Jones and Takai, 2001). As these CpG dinucleotides are palindromic, both DNA strands are typically methylated at CpGs (mCG), which allows for the conservation of DNAm patterns during cell division (Jones, 2012). DNAm can also occur in low levels at CpH sites (mCH; where H = A, C, or T), particularly within neuronal cells of the brain (Lister et al., 2013; Guo et al., 2014). DNAm patterns are established and maintained by DNA methyltransferases (DNMTs), a highly conserved family of enzymes that includes DNMT1 (maintenance), as well as DNMT3a and DNMT3b (establishment) (Denis et al., 2011).

Importantly, DNAm is associated with gene expression, although its effects on transcriptional regulation depend on genomic context (Jones and Baylin, 2007; Lam et al., 2012; Edgar et al., 2014). For instance, DNAm in promoter regions is associated with gene expression silencing, while its role is more variable within gene bodies (Jones and Baylin, 2007; Schuebeler, 2015). Conversely, in regions of lower CpG density, high DNAm levels are typically correlated with highly expressed genes, especially if the associated CpG island is lowly methylated (Irizarry et al., 2008; Baubec and Schuebeler, 2014; Edgar et al., 2014). Although the underlying mechanisms of transcriptional silencing by DNAm remain elusive, they may potentially act through the direct inhibition of transcription factors or the recruitment of transcriptional repressors to *cis*-regulatory regions (Tate and Bird, 1993). In addition to its role in the regulation of gene expression and cellular programs, DNAm is emerging as a potential biomarker for environmental exposures and various disorders thanks to its temporal stability and responsivity to external influences (Bock, 2009).

By contrast, DNAhm of cytosine residues (hmC) may act as an intermediate in the demethylation cycle of DNA by the Ten-Eleven Translocation (TET) family of enzymes, and may also play a role in the transcriptional regulation by recruiting different chromatin modifiers (Sadakierska-Chudy et al., 2014). Of note, high levels of DNAhm are found in pluripotent cells and the brain, where it has been associated with the cellular functions of neural stem cell (Kriaucionis and Heintz, 2009; Ito et al., 2010; Santiago et al., 2014). Genome-wide analysis of these modifications in the frontal cortex, hippocampus, and cerebellum identified higher levels of DNAhm in gene bodies, which were positively correlated with gene expression in developmentally active genes (Wang et al., 2012; Lister et al., 2013). Furthermore, TET activity and subsequent active DNA demethylation are associated with memory formation and addiction in mice, suggesting a key role in neural activity and function (Alaghband et al., 2016). Finally, additional oxidized cytosine base variants have been identified, including formylcytosine and carboxycytosine, although their functional roles have not yet been characterized in neural tissues (Ito et al., 2011).

Methods for assessing DNA modifications

Different approaches have been used to study DNA modification patterns, ranging from bulk levels to targeted and genome-wide techniques. Methods to investigate bulk levels of DNA modifications range from immunoblotting and fluorescence methods to the analysis of repetitive elements to obtain a snapshot of levels across the entire genome (Harrison and Parle-McDermott, 2011; Sun et al., 2015). However, these are now being superseded by genome-wide methods, which provide

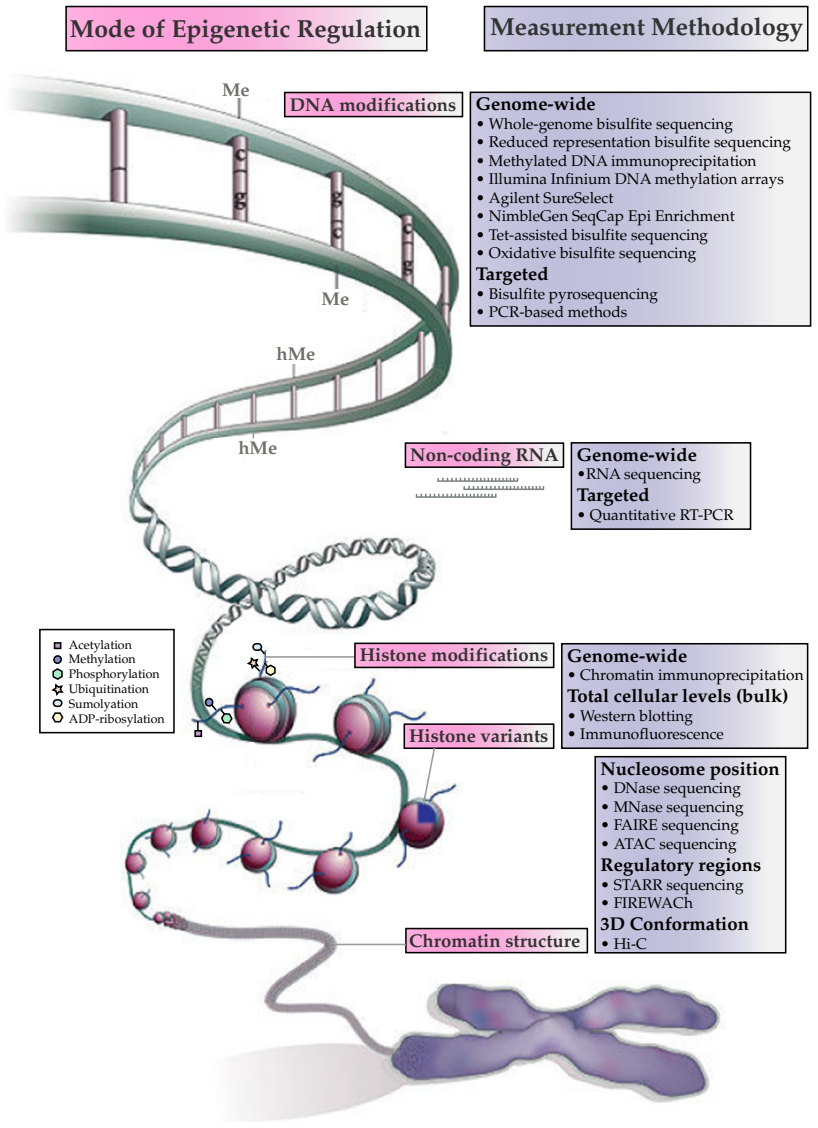

Fig. 17.1. The predominant modes of epigenetic regulation and the different methodologies used to assess them. The epigenome is comprised of various levels of regulatory processes involving DNA modifications, noncoding RNA, histone modifications, and histone variants, as well as chromatin structure. Here, we depict these different forms of epigenetic regulation (pink boxes) and list the methodologies and techniques used to measure them (purple boxes). Note that a representative selection of modifications is illustrated in this figure, but others do exist (i.e., formyl or carboxyl modifications for DNA; biotinylation for histones); please see text for detailed discussion. *hMe*, hydroxymethylation; *Me*, methylation; *PCR*, polymerase chain reaction; *RT-PCR*, reverse transcription polymerase chain reaction. (Adapted from Qiu J (2006) Unfinished symphony. Nature 441: 143-145. Copyright © 2006, Nature Publishing Group (license number: 417035112252). with permission.)

additional insight into the biologic underpinnings of environmental exposures and disease.

For the most part, genome-wide methods rely on the chemical treatment of cytosine residues to obtain DNA modification information. Bisulfite conversion is the primary method used to investigate DNA methylation, converting unmodified cytosines to uracil, while leaving cytosines with DNA modifications unaffected. However, this technique also results in the protection of hydroxymethylated residues, and the resulting uracil/cytosine ratio reflects both types of modification. As such, additional biochemical treatments have been developed to deconstruct this confound, including TET-assisted bisulfite (TAB) and oxidative bisulfite conversion, which only protect hmC from conversion to uracil (Yu et al., 2012a; Booth et al., 2013). These are being used in conjunction to identify the ratio of DNA modifications at a given cytosine residue.

Several techniques are currently being used in the genome-wide analysis of DNA modifications using the above methods. The current gold standard, whole-genome sequencing, provides DNA modification information for all sites within the genome, but requires high sequencing coverage and results in a large proportion of unused genetic information (Lister et al., 2009). By contrast, other techniques use enrichment approaches to limit analyses to regions of the genome containing epigenetic information. Reduced representation bisulfite sequencing uses methylation-sensitive enzymes to digest DNA and size selection to enrich regions of high CpG density for sequencing (Meissner et al., 2005; Boyle et al., 2012). This results in lower overall coverage of the genome and an enrichment of CpG islands and promoters, which may be less sensitive to environmental influences, but are more closely linked to gene expression patterns.

Enrichment can also be performed through capture methods, such as Agilent's SureSelect human methyl-seq or NimbleGen's SeqCap epi enrichment system, which use custom-designed oligonucleotides to select relevant regions of the genome. In addition to these sequencing-based approaches, commercial arrays from Illumina (GoldenGate (∼1500 sites), 27K (∼27,000 sites), 450K (∼485,000 sites) and currently the EPIC (∼860,000 sites)) provide quantitative DNAm or DNAhm data across the genome, and are the most commonly used method to investigate DNAm changes in human populations (Bibikova et al., 2006, 2009, 2011; Moran et al., 2016). Furthermore, more targeted methods, such as pyrosequencing, also rely on these chemical conversions to obtain quantitative epigenetic data at specific loci. In contrast to these methods, methylated DNA immunoprecipitation uses antibodies raised against DNA methylation or hydroxymethylation to select regions of the genome enriched for these DNA modifications (Maunakea et al., 2010; Nair et al., 2011; Pälmke et al., 2011; Taiwo et al., 2012). This method is unbiased towards CpG-rich regions, representing a robust approach to analyze genome-wide DNAm patterns, while simultaneously reducing the complexity of the data set by omitting unmethylated regions.

Chromatin

In the cell, DNA is bound to a dynamic nucleoprotein polymer known as chromatin, which is regulated by various factors that allow for DNA compaction within the nucleus and modulate access to genetic material. The fundamental repeating unit of chromatin is the nucleosome, consisting of 147 base pairs of DNA wrapped around a protein octamer containing two molecules each of core histone proteins H2A, H2B, H3, and H4 (Kornberg, 1974; Luger et al., 1997).

The flexible N-terminal tails of histones are targets for post-translational modifications, which regulate chromatin structure and transcription by altering the accessibility of the local chromatin structure, serving as docking sites for various effector proteins and chromatin-remodeling complexes, and modulating the binding of transcription factors (Venkatesh et al., 2013). Histone modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation, which assume different functional roles based on their cellular context and position on the histone tail (Kouzarides, 2007; Bannister and Kouzarides, 2011; Bowman and Poirier, 2015). In general, histone acetylation and histone H3 lysine 4 trimethylation (H3K4me3) are generally associated with increased transcription, while trimethylation of histone H3 lysine 9 (H3K9me3) and 27 (H3K27me3) is correlated with transcriptional repression.

Canonic histones can also be exchanged for different variants, such as H2A.Z and H3.3, which are crucial to the regulation of chromatin neighborhoods and can influence nucleosome structure, stability, dynamics, and, ultimately, gene expression and cellular function (Weber and Henikoff, 2014). These chromatin alterations are mediated by chromatin-remodeling enzymes, which are typically found in multiprotein complexes that regulate the chromatin landscape through the incorporation of histone variants or addition of histone modifications (Cairns, 2007; Narlikar et al., 2013).

Importantly, histone variants and modifications also play a role in mediating the two main types of chromatin structure found in the nucleus: highly condensed regions known as heterochromatin, and relatively open and more accessible conformation known as euchromatin (Allis and Jenuwein, 2016). Euchromatin typically contains actively transcribed genes, while heterochromatin mainly contain transcriptionally silenced elements (Grewal and Jia, 2007; Allis and Jenuwein, 2016). Given that chromatin structure and transcriptional activity are tightly linked processes, their relationship requires a dynamic interplay between histone modifications, histone variants, and chromatin-remodeling complexes (Deal and Henikoff, 2010).

Methods for the investigation of chromatin structure and modifications

Similar to DNA modifications, methods for the investigation of histone modifications structure range from bulk levels to genome-wide patterns. These are heavily based on antibody specificity, where immunohistochemistry and protein blots are used to assay bulk levels, while chromatin immunoprecipitation (ChIP) is used to pull down regions of the genome associated with specific

marks (Lara et al., 2011). In turn, the latter can be combined with quantitative polymerase chain reaction (ChIP-qPCR) to assay levels at specific loci, or with next-generation sequencing (ChIP-seq) to obtain genome-wide enrichment information. This method can also be used to analyze the genomic distribution of different histone variants, transcription factors, or other chromatin-associated proteins and epigenetic modifiers.

Nucleosome positioning and chromatin states (euchromatin versus heterochromatin) can be assayed through a spectrum of methods that use the sensitivity of DNA to enzymatic digestion (DNase-seq; MNase-seq), formaldehyde linking (FAIRE-seq), or transposable element incorporation (ATAC-seq) to identify open regions of chromatin (Zentner and Henikoff, 2014). The mapping of regulatory regions can be taken one step further through the identification of enhancer regions using self-transcribing active regulatory region sequencing (STARR-seq) and functional identification of regulatory elements within accessible chromatin (FIREWACh) (Arnold et al., 2013; Murtha et al., 2014). Finally, the three-dimensional conformation of chromatin can be assessed through cross-linking experiments and whole-genome sequencing, providing insight into *trans*- and *cis*-acting regulatory regions of the genome (Lieberman-Aiden et al., 2009). Importantly, several of these techniques require fresh tissue for high-quality data, limiting their relevance in the context of postmortem studies.

Noncoding RNA

ncRNAs represent an additional layer of epigenetic regulation and are distinct from messenger RNA (mRNA) in that they are not translated into protein. These untranslated RNA species regulate a wide number of cellular functions, ranging from mRNA and protein level regulation to the inhibition of the repetitive and transposable elements of the genome. In particular, microRNAs (miRNAs) inhibit the translation of mRNA into protein, tempering the levels of the different cellular effectors. In addition, long ncRNAs (lncRNAs) are important regulators of the cell, acting as guides for epigenetic repression or activation, decoys for chromatin-modifying enzymes or RNA interference (RNAi) pathways, and regulators of post-transcriptional RNA processing (Rinn et al., 2007; Orom et al., 2010; Wang and Chang, 2011; Wutz, 2011; Geisler and Coller, 2013). These transcripts are also crucial developmental, spatial, temporal, and stimulus-specific cues required for the integration of complex gene expression and epigenetic patterns in the brain (Wang and Chang, 2011). Furthermore, neurons express very high levels of ncRNAs and altered expression of several ncRNA transcripts is associated with different neurologic disorders, including autism, fragile X, Rett syndrome, schizophrenia, and anxiety-like disorder (Millar et al., 2000; Gustincich et al., 2006; Williams et al., 2009; Kapranov et al., 2010; Spadaro and Bredy, 2012; Barry et al., 2013; Petazzi et al., 2013; Ziats and Rennert, 2013; Pastori et al., 2014; Spadaro et al., 2015).

Methods for ncRNA analysis

Methods to investigate ncRNA levels are similar to those used to assess mRNA levels, though they occasionally require additional selection methods and greater sequencing depth to detect low-abundance isoforms. Various microarrays have been developed to analyze known ncRNA, including different miRNA and lncRNA, and provide a cost-effective method to assess a wide variety of ncRNA. By contrast, RNA-sequencing can be used to assay all ncRNA species in the cell in a highly quantitative manner, though size selection is often required in the case of miRNA (Landgraf et al., 2007). In addition, this method can potentially detect splice variants with high sequencing depth. Finally, reverse-transcription quantitative PCR is frequently used to investigate specific ncRNA species in a targeted manner, and represents an important validation method for microarray and next-generation sequencing methods.

OVERVIEW OF EXISTING EPIGENETIC STUDIES USING POSTMORTEM BRAIN

Epigenomic variation in postmortem human brain tissues has provided compelling insight into the complex relationship between epigenetic regulation and genome function in the context of brain development and health. Spanning a diverse range of fields from developmental to neurologic to psychiatric disease research, this burgeoning body of work has garnered widespread interest for the role of epigenetic mechanisms in brain-related phenotypes amongst the scientific community and the public alike. In this section, we provide a detailed overview of existing epigenetic studies using postmortem brain in the context of: (1) normative brain function and development; (2) neurodevelopmental disorders; (3) psychiatric disorders; and (4) neurodegenerative diseases.

Characterization of the epigenomic landscape in healthy postmortem brain tissue

Profiling DNA modifications in healthy postmortem adult brain

Insights into gene-regulatory mechanisms underlying typical brain function and development have been largely spurred by the generation of genome-wide epigenomic profiles in healthy brain tissues. To date, a variety of epigenetic marks have been profiled across the human

brain at the nucleotide level, yielding a dynamic map of epigenomic variation in neural tissues. In the context of DNA modifications, genome-wide profiling studies of the human adult brain provide an intriguing view of the neuronal methylome in terms of its unique and complex patterns of mCG, mCH, and hmC marks. Specifically, mCG patterns in postnatal neurons of the prefrontal cortex and hippocampus are largely similar to other cell types in that intergenic regions and repeat DNA exhibit high levels of mCG, while active regulatory elements, such as enhancers and promoters, show mCG depletion (Lister et al., 2009, 2013; Ziller et al., 2013). These findings suggest that neuronal mCG plays a key role in transcriptional silencing of DNA repeats across the genome and the regulation of gene expression by transcriptional repression (Suzuki and Bird, 2008; Smith and Meissner, 2013).

For non-CpG methylation, the distribution and functional roles of mCH can vary by cell type. Although, mCH is nearly absent in nonneuronal adult somatic cells, mCH constitutes the predominant form of cytosine methylation in adult neurons, with mCH accounting for ~53% of the total methylated fraction of adult neuronal genome and occurring mainly in CAC trinucleotides (Ziller et al., 2011; Lister et al., 2013; Guo et al., 2014). Similar to mCG, neuronal mCH largely serves to repress gene transcription and is depleted from expressed genes with levels in 5'-upstream, gene body, and 3'-downstream regions being inversely correlated with transcript abundance (Lister et al., 2013; Guo et al., 2014). However, mCH is not clearly associated with gene repression in all cell types as it has been shown to positively correlate with gene transcription in embryonic stem cells, suggesting that the distinct functional role(s) of mCH may relate to the unique, tissue-specific complement of DNAm writers and readers in a cell (Ziller et al., 2011; Kinde et al., 2015).

In addition to canonical forms of DNAm, the advent of high-throughput techniques such as TAB-seq has enabled the quantification of cytosine modification variants (i.e., DNAhm) at base resolution in human brain tissues (Yu et al., 2012b; Chopra et al., 2014). Such analyses have shown that hmC is substantially enriched in neurons of the frontal cortex and cerebellum, with 10-fold greater level of hmC in the brain over embryonic stem cells (Lister et al., 2013; Wen et al., 2014). With most hmC in the brain occurring in the CG context, hmCG exhibits opposing distribution patterns to canonical mCG in human frontal cortex and cerebellum, including depletion from transcriptional start sites and selective enrichment throughout intragenic regions of highly expressed genes and active regulatory elements of intergenic regions (Szulwach et al., 2011; Lister et al., 2013; Wen et al., 2014). In this regard, hmC has been implicated as an intermediate in the active demethylation pathway, although various cellular factors can bind hmCG, suggesting that this cytosine variant serves as a stable neuronal epigenetic mark in its own right (Spruijt et al., 2013; Bachman et al., 2014). Together, such high-throughput mapping analyses present a compelling portrait of the diverse composition, distribution, and functional roles of DNA modifications in the adult human brain epigenome.

Profiling DNA modifications in fetal brain tissues

In addition to profiling the methylome of adult neural tissues, there has been substantial focus on characterizing the dynamic regulation of DNA modifications in the developing fetal brain (Gabel and Greenberg, 2013). Earlier DNAm array-based work using dorsolateral prefrontal cortex tissue ranging from prenatal development to late life showed extensive age-related alterations in DNAm, although these analyses were largely focused on promoter-associated regions (Numata et al., 2012). Subsequent sequencing work performed in frontal cortex tissue from the developing brain at key postnatal stages reported significant accumulation of non-CpG methylation in neurons during early postnatal development, with rapid increases during the primary phase of synaptogenesis (0–2 years in humans) (Lister et al., 2013). While glia exhibit low total levels of mCH, there is selective enrichment of mCH in genes associated with brain cell type specification, suggesting that mCH may enforce cell lineage-specific transcriptional profiles (Lister et al., 2013). More recently, a genome-wide DNAm analysis using the largest cohort of fetal brain tissues to date ($n = 179$), which ranged from 23 to 184 days postconception, showed highly significant DNAm changes across fetal brain development at ~29,000 sites, with an enrichment of CpGs losing methylation with fetal age (Spiers et al., 2015). These sites, which were largely associated with genes involved in neurodevelopmental processes, were significantly underrepresented in promoter regions but overrepresented in gene bodies and regions flanking CpG islands (i.e., shores and shelves) (Spiers et al., 2015).

In regard to DNAhm, hmC also builds up in neurons postnatally, coinciding with a period of active synaptic maturation, and selective localization to cell type-specific enhancers (Lister et al., 2013). Using an oxidative bisulfite treatment of genomic DNA prior to Illumina 450K array analysis, a recent study was able to quantify hmC levels in the fetal brain epigenome, reporting overall low levels of global DNAhm with ~300,000 autosomal sites exhibiting detectable hmC, which showed substantial interindividual variation (Spiers et al., 2017). DNAhm sites that changed during fetal brain development were significantly depleted in

promoter-associated regions, including CpG islands, and formed detectable co-hydroxymethylated modules localized to genes involved in neurodevelopmental processes (Spiers et al., 2017). Taken together, these studies showcase the dynamic variation of mCG, mCH, and hmC marks in the developing fetal brain of humans, further supporting the role of epigenetic processes in influencing early-life neuronal plasticity as well as neurobiologic and cognitive functions across the life course (Borrelli et al., 2008; Day and Sweatt, 2010; Ma et al., 2010; Guo et al., 2011).

Integration of DNA modifications and genetic variation in the postmortem brain

A growing area of interest in the field of population-based epigenetic studies is the association of genetic variation with DNAm, at sites known as methylation quantitative trait loci (mQTLs) (Jones et al., 2013). Such analyses have been performed in various adult brain regions, including the cerebellum, frontal cortex, temporal cortex, and pons, finding that proximally located single-nucleotide polymorphism (SNP)-CpG pairs (known as *cis*-mQTLs) are more predominant than distally located SNP-CpG pairs (known as *trans*-mQTLs) (Gibbs et al., 2010; Zhang et al., 2010).

More recent work linking underlying genetic architecture to DNAm variation in the developing fetal brain showed that genetic effects on fetal brain methylome were largely stable across the lifespan and exhibited a significant enrichment of psychosis-associated risk loci (Hannon et al., 2016; Jaffe et al., 2016). Cross-tissue comparisons of mQTLs between peripheral blood and four different brain regions (temporal cortex, frontal cortex, pons, and cerebellum) showed significant overlap (18.5–31.6%) between the tissues, with the highest proportion of overlap observed between brain regions (35.8–71.7%), although the DNAm change per allele differed between tissues (Smith et al., 2014).

Finally, the first study to explore genetic influences on DNAhm in the brain reported 23 significant hydroxymethylation quantitative trait loci passing a genome-wide Bonferroni significance threshold in fetal cortex samples, despite the fact that their analyses were likely limited by small sample size and the relative scarcity of hmC marks (Spiers et al., 2017). Collectively, these findings help shed new light on the relationships between sequence polymorphisms and DNA modification variants, particularly in terms of their wide-reaching biologic relevance and their potential to contribute to interindividual variation in gene regulation.

Mapping histone modifications in the epigenome of healthy brain tissues

Genome-wide mapping of histone modifications in postmortem brain samples has greatly expanded our understanding of chromatin biology as it relates to neural function and development. Early work used human cerebellar cortex samples collected across a wide age range from mid-gestation to 90 years of age to assess the distribution of histone lysine methylation at various candidate loci, namely promoter regions of 16 ionotropic and metabotropic glutamate receptor genes (Stadler et al., 2005). This study identified substantial development-related and gene-specific differences in H3K4me2, H3K4me3, H3K27me3, and H3K20me3 marks, along with robust correlations with mRNA levels of cognate genes (Stadler et al., 2005).

More recent methodologies have refined the characterization of histone post-translational modifications in bulk postmortem brain samples, allowing researchers to profile histone modifications in neuronal epigenomes from heterogeneous brain tissues through antibody-based targeting of the neuron-specific NeuN marker in conjunction with ChIP-seq (Huang et al., 2006; Kundakovic et al., 2017). Genome-wide mapping of H3K4me3 in neuronal and nonneuronal nuclei collected from prefrontal cortex tissues across a wide age range revealed dramatic and widespread alterations in H3K4me3 patterns in the developing prefrontal neurons, with less extensive changes occurring in mature neurons later in life (Cheung et al., 2010). In addition, combinatorial mapping of multiple chromatin marks (H3K4me1, H3K4me3, H3K9me3, H3K27me3, H3K36me3, H3K9ac, and H3K27ac) across 29 different primary tissues and cell types, including six distinct brain regions (anterior caudate, cingulate gyrus, hippocampus, inferior temporal lobe, midfrontal lobe, and substantia nigra) revealed diverse regulatory functions of histone post-translational modifications across different developmental stages, lineages, and cellular states (Zhu et al., 2013).

Finally, more recent efforts have sought to integrate H3K9Ac ChIP-Seq and genotyping data in one of the largest cohorts of dorsolateral prefrontal cortex samples ($n = 494$), identifying histone acetylation quantitative trait loci which exhibit both independent and shared effects with gene expression (Ng et al., 2017). Together, such high-throughput profiling of the histone post-translational modification landscape in neuronal epigenomes of postmortem brain provides valuable opportunities to more deeply characterize brain-specific chromatin structure and function, particularly in terms of their impact on gene regulation.

Characterization of noncoding RNA species in healthy postmortem brain

Transcriptomic profiling of ncRNAs in human postmortem brain has also shed new light on the role of ncRNAs as key post-transcriptional regulators of gene expression,

implicating them in various processes that are central to neural function, including cell type specification, neurite projection, and synaptic plasticity (Im and Kenny, 2012). Previous work assessing miRNA expression levels in prefrontal cortex and cerebellar cortex samples from healthy donors across a wide age range found substantial age-related miRNA variation and specifically identified miR-92a, miR-454, and miR-320b as possible regulators of human-specific neural development (Somel et al., 2010, 2011). Analyses of microRNA levels from numerous brain regions across various developmental stages further reported that the greatest variation in miRNA expression occurs during the transition from infancy to early childhood, with the dorsolateral prefrontal cortex exhibiting the greatest number of differentially expressed miRNAs, followed by the cerebellum and the hippocampus (Moreau et al., 2013; Ziats and Rennert, 2014). Furthermore, transcriptome-wide profiling of RNA interactions with Ago2, a core protein component of miRNA-silencing complexes, identified nearly 7000 putative miRNA-binding sites in adult motor cortex and cingulate gyrus (Boudreau et al., 2014). These findings not only further substantiate the contribution of miRNAs in brain development and function, but also serve as a valuable resource for exploring cross-talk and broader regulatory networks for miRNA activity (Boudreau et al., 2014).

In addition to miRNA species, accumulating evidence suggests that lncRNA species may not simply arise from "transcriptional noise" from existing protein-coding genes, but may rather serve distinct functional roles (van Bakel et al., 2010; Roberts et al., 2014). The latter hypothesis is supported by a landmark study, which reported strong statistical associations between lncRNA expression and brain development in human cerebral cortex tissue, as well as by the finding by the GENCODE consortium that the majority of lncRNAs are independent transcriptional units (Derrien et al., 2012; Lipovich et al., 2014). Taken together, these results implicate ncRNA as key contributors to the complex regulatory architecture underlying healthy brain development and function across the lifespan.

Assessment of epigenomic variation associated with neurodevelopmental diseases

The etiologic basis for a number of neurodevelopmental disorders involves the disruption of genes encoding epigenetic factors (reviewed in Zahir and Brown, 2011). For example, mutations in the X-linked gene *MECP2*, which encodes a methyl-CpG-binding protein, causes Rett syndrome, a childhood disorder associated with a broad range of developmental, cognitive, and neurologic deficits (Amir et al., 1999). Loss-of-function mutations in the DNA methyltransferase gene *DNMT3B* are responsible for immunodeficiency, centromere instability, facial anomalies (ICF) syndrome, a disorder involving psychomotor retardation and defective brain development (Hansen et al., 1999; Ehrlich et al., 2006).

Another common group of neurodevelopmental disorders is autism spectrum disorders (ASD), which are characterized by severe impairments in social interaction, communication and behavioral patterns that are restrictive and stereotypical (Rapin, 1997). Increased interest in epigenetic profiling in ASD samples has been prompted by the observation that there is notable overlap in clinical features and symptoms between ASD and other neurodevelopmental disorders, such as Angelman, fragile X, and Rett syndromes, which are caused by epigenetic perturbations or mutations in genes encoding epigenetic factors (Samaco et al., 2005). Candidate gene DNAm studies in ASD using superior temporal gyrus and cerebral cortex samples previously reported increased promoter DNAm at the *RELN* and *MECP2* genes, respectively (Nagarajan et al., 2006; Lintas et al., 2016). On a genome-wide level, interrogation of DNAm in ASD dorsolateral prefrontal cortex, temporal cortex, and cerebellum samples using the Illumina 450K array revealed four differentially methylated regions, which reached genome-wide significance, with three out of four differentially methylated regions replicating in an independent cohort (Ladd-Acosta et al., 2014).

In the context of histone post-translational modifications, neuronal-specific profiling of H3K4me3 marks in prefrontal cortex showed ASD-related alterations in H3K4me3 occupancy at numerous genes associated with neuronal connectivity, social behaviors, and cognition, including previously described ASD genetic risk loci (Shulha et al., 2012a). Recently, a histone acetylatome-wide association study, measuring H3K27ac marks by ChIP-Seq, was conducted in postmortem prefrontal cortex, temporal cortex, and cerebellum tissues from ASD individuals and age-matched controls (Sun et al., 2016). This study found that, despite clinical heterogeneity, $>$ 68% of ASD cases shared a common acetylome signature at $>$ 5000 *cis*-regulatory elements genome-wide, with ASD-related H3K27ac aberrations occurring at genes associated with synaptic transmission, ion transport, epilepsy, and behavioral abnormality (Sun et al., 2016). Moreover, by correlating histone acetylation with genotype, this study additionally uncovered $>$2000 histone acetylation quantitative trait loci in human brain regions, signifying genetic influences on acteylome variation (Sun et al., 2016). Collectively, these epigenetic analyses in ASD postmortem brain not only provide a rich resource for further molecular studies in ASD, but also serve as a foundation for future high-throughput epigenetic investigations in postmortem samples of other neurodevelopmental disorders.

Interrogation of epigenomic alterations associated with psychiatric disorders

Profiling DNA modifications in postmortem brain tissues with neuropsychiatric disorders

Increasing interest in epigenomic contributions to complex, environmentally-conditioned neuropsychiatric diseases has been potentiated by the growing recognition that epigenetics may serve as a mediator between genomic variation and environmental influences (Connor and Akbarian, 2008; Bredy et al., 2010; Klengel and Binder, 2015; Nestler et al., 2015; Fullard et al., 2016). Specifically, epigenetic associations have been investigated for a number of different psychiatric conditions, including schizophrenia, bipolar disorder, major depressive disorder (MDD), and various addiction disorders (Connor and Akbarian, 2008; Bredy et al., 2010; Nestler et al., 2015).

In the context of DNA modifications, candidate gene analyses in postmortem brain have reported significant DNAm alterations associated with schizophrenia and bipolar disorder at specific loci, including *RELN*, *COMT*, *SOX10, HTR2A, HTR1A, BDNF, HCG9, KCNQ3*, and *DAT1* (Abdolmaleky et al., 2005, 2006, 2011; Grayson et al., 2005; Carrard et al., 2011; Kaminsky et al., 2012, 2015; Kordi-Tamandani et al., 2012; Pal et al., 2015). However, these candidate DNAm associations have not been consistently replicated, likely owing to differences in methodology, the specific CpG dinucleotides interrogated, brain regions examined, and clinical populations from which the postmortem samples were collected (Dempster et al., 2006; Tamura et al., 2007; Tochigi et al., 2008).

In order to achieve more comprehensive and unbiased assessments of DNAm variation in major psychosis, one of the earliest genome-wide analyses of psychosis used CpG island micorarrays to analyze postmortem frontal cortex tissue from schizophrenic, bipolar disorder, and control subjects (Mill et al., 2008). The study identified psychosis-associated DNAm alterations in genes related to glutamatergic and GABAergic neurotransmission, neuronal development, and metabolism (Mill et al., 2008). These results were further corroborated by a subsequent finding that *DNMT1* and *TET1*, two genes encoding enzymes that respectively methylate and hydroxymethylate CpGs, were overexpressed in the brain of schizophrenic and bipolar disorder patients and that DNMT1 showed increased binding to a subset of GABAergic (i.e., *GAD1*) and glutamatergic (i.e., *BDNF*) gene promoters in the cerebral cortex, but not the cerebellum (Dong et al., 2015).

More recent genome-wide DNAm profiling using commercial microarrays, namely Illumina 27K and 450K arrays, in prefrontal cortex, dorsolateral prefrontal cortex, frontal cortex, cerebellum, and hippocampus tissues have reported widespread DNAm aberrations associated with schizophrenia at numerous genes involved in neurodevelopmental processes and GABAergic neurotransmission, including a large number of *cis*-mQTLs which overlapped with risk SNPs implicated in schizophrenia (Chen et al., 2014; Numata et al., 2014; Pidsley et al., 2014; Wockner et al., 2014; Hannon et al., 2015; Jaffe et al., 2015; Ruzicka et al., 2015; Gagliano et al., 2016). Taken together, these findings implicate DNAm alterations related to early neurodevelopmental programs and neurotransmission regulation, along with genetic risk variants, as potential contributors to schizophrenia and bipolar disorder pathophysiology.

In addition to schizophrenia and bipolar disorder, DNA modifications have also been assessed in the context of MDD and addiction disorders. Specifically, high-throughput profiling of ~3.5 million CpGs in postmortem frontal cortex MDD samples identified 244 MDD-associated differentially methylated regions which were highly enriched for neuronal growth and development genes (Sabunciyan et al., 2012). Hippocampal DNAm differences in male offspring were also observed in relation to maternal depression during pregnancy as well as increased DNAm at specific loci (i.e., *BDNF*) in Wernicke area in relation to suicidal behavior, signifying epigenetic associations with a broad range of depression-related behavioral phenotypes (Keller et al., 2011; Nemoda et al., 2015).

Recently, DNAhm variation was assessed in postmortem prefrontal cortex samples of depressed individuals and, although no individual hmC site reached genome-wide significance, 550 CpGs with suggestive evidence of differential hydroxymethylation were identified (Gross et al., 2017). In regard to addiction disorders, co-methylated modules enriched for genes involved in neural development and transcriptional regulation were identified in prefrontal cortex tissues of male alcohol-dependent patients (Wang et al., 2016). In addition, *cis*-mQTLs underlying the prodynorphin gene (*PDYN*) were associated with alcohol dependence status in dorsolateral prefrontal cortex tissues (Taqi et al., 2011). Overall, these studies provide compelling evidence for the potential role of brain-specific DNA modifications in moderating the combined contribution of genetic and environmental factors in neuropsychiatric disorders.

Investigation of chromatin alterations in postmortem brain tissues associated with neuropsychiatric disorders

Assessments of histone post-translational modification distributions in postmortem psychiatric brain samples have further deepened our molecular understanding of neuropsychiatric disorders. Quantification of bulk levels

of phosphorylation, acetylation, and methylation at various H3 and H4 residues in postmortem prefrontal cortex of schizophrenic patients showed significant increases in levels of H3-(methyl)arginine 17 (H3meR17), commensurate with downregulated expression in a subset of metabolic genes (Akbarian et al., 2005). Bulk levels of H3K9me2, measured by protein blot, were also higher in parietal cortex tissue of schizophrenia individuals over controls in a manner that is positively correlated with expression of histone methyltransferase genes *GLP* and *SETDB1* (Chase et al., 2013).

Characterization of H3K4 and H3K27 trimethylation in prefrontal cortex tissues from schizophrenic patients also revealed a shift from H3K4me3 to H3K27me3 at key genes involved in GABAergic neurotransmission regulation, including *GAD1, GAD2, NPY*, and *SST*, over matched controls (Huang et al., 2007). This transition from open to repressive chromatin marks at these genes was concomitant with decreased *GAD1* expression and influenced by the presence of schizophrenia-associated risk alleles in *GAD1* (Huang et al., 2007). Simultaneous profiling of chromatin marks and DNAm at candidate CpGs in the *GAD1* gene showed that repressive chromatin (i.e., H3K27me3 enrichment) exhibited coinciding DNAm alterations in *GAD1* promoter CpGs in schizophrenic prefrontal cortex over matched controls; however, this trend was not observed in open chromatin (i.e., H3K4me3-enriched) regions (Huang and Akbarian, 2007).

In the context of MDD, chromatin profiling in nucleus accumbens of depressed subjects showed enrichment of H3K27me3 in the *Rac1* gene, encoding a factor involved in synaptic structure modulation, accompanied with decreased transcription of *Rac1* (Golden et al., 2013). In regard to addiction disorders, ChIP-seq analysis of H3K4me3 levels in the hippocampus of both cocaine addicts and chronic alcohol abusers showed significant overlap in H3K4me3 profiles, with greater effects detected for cocaine addiction, suggesting neuronal adaptations that may be common to both addictions (Zhou et al., 2011). Taken together, these findings not only constitute a preliminary framework for the study of chromatin biology in other psychiatric syndromes, but also provide some of the first examples for how histone post-translational modifications could contribute to psychosis-related gene expression aberration and neural dysfunction.

Assessment of noncoding RNA patterns in postmortem brain of neuropsychiatric cases

Accumulating evidence suggests that neuronal ncRNA dysregulation likely contributes to the development and progression of psychiatric diseases (Kocerha et al., 2015). Early high-throughput microRNA expression analysis in cortical gray matter from the superior temporal gyrus of schizophrenia subjects showed significant upregulation of miR-181b over controls (Beveridge et al., 2008). Subsequent quantification of miRNAs in prefrontal cortex samples from a cohort of schizophrenia and bipolar disorder patients found significant psychosis-associated differential expression of numerous miRNAs, which targeted brain-specific genes involved in neurodevelopment, behavior, and synaptic plasticity (i.e., miR-132) (Moreau et al., 2011; Kim et al., 2012; Miller et al., 2012). Postmortem profiling of miRNA expression in the prefrontal cortex of schizophrenia patients revealed global increases in miRNA expression, largely pertaining to mature and pre-miRNA species with no changes in source pre-miRNA, over control samples (Perkins et al., 2007; Beveridge et al., 2010; Santarelli et al., 2011). These findings suggest that the schizophrenia-related alterations are due to changes in miRNA biogenesis rather than altered miRNA transcription (Beveridge et al., 2010).

Deficits in miRNA biogenesis were also indicated by results from a study that assessed miRNA expression in prefrontal cortex samples of a well-characterized cohort of MDD, bipolar, and schizophrenia subjects (Smalheiser et al., 2014). Specifically, alterations in levels of discrete miRNAs were observed in all disorders, as well as in suicide completers from each of the diagnostic categories, compared to controls (Smalheiser et al., 2014). Interestingly, downregulated miRNAs associated with schizophrenia were enriched at synapses, while upregulated miRNAs were not, suggesting that schizophrenia-related alterations in miRNA processing are largely localized in the synaptic compartment (Smalheiser et al., 2014).

Finally, in addition to miRNA dysregulation, aberrant decreases in lncRNA species, specifically lncRNA *Gomafu*, along with associated splicing defects, have been reported in cortical gray matter from the superior temporal gyrus of schizophrenic patients over controls (Barry et al., 2014). Intriguingly, four lncRNAs, MIAT, MEG3, NEAT1, and NEAT2, were upregulated in the nucleus accumbens of heroin users, suggesting a role for lncRNA dysregulation in addiction disorders (Michelhaugh et al., 2011). Taken together, these findings suggest that disruptions in ncRNA activity may contribute to psychiatric disease pathogenesis, likely through disorganization of larger regulatory networks in the neuronal epigenome.

Characterization of epigenomic changes associated with neurodegenerative diseases

Profiling DNA modifications in neurodegenerative postmortem brain tissues

Studies of DNA modifications in the postmortem brain of neurodegenerative disorders have become increasingly

prevalent due to their potential to provide insights into potential molecular underpinnings of these degenerative brain disorders (Landgrave-Gómez et al., 2015). Specifically, DNA modifications have been assessed in the context of postmortem brain samples from Huntington disease (HD), Alzheimer disease (AD), and Parkinson disease (PD).

In the case of HD, early work using methylated DNA immunoprecipitation in postmortem putamen specimens reported increased mC and reduced hmC levels in the 5'UTR of a candidate gene, *ADORA2A,* of HD patients over age-matched controls (Villar-Menéndez et al., 2013). By contrast, more recent DNAm profiling using the Illumina 450K array in postmortem frontal cortex samples of HD patients and age-matched controls revealed minimal evidence of HD-associated DNAm differences at probed sites after correction for cell type heterogeneity, but did report an association between HD cortical DNAm variation and age of disease onset (De Souza et al., 2016). Moreover, by comparing DNAm profiles between matched postmortem cortex and liver tissues, this study identified a tissue-specific DNAm signature underlying the huntingtin (*HTT*) gene promoter, which encompasses a differentially methylated binding site for the transcription factor CTCF (De Souza et al., 2016). Such findings illustrate how DNAm profiling in postmortem brain can help illuminate potential epigenetic regulation underlying tissue-specific expression patterns.

DNAm alterations have also been implicated in AD brain pathology, with early immunoblotting analyses showing reductions in global mC and hmC from entorhinal cortex, temporal neocortex, and hippocampus of AD patients over controls, although attempted replications of these findings have produced conflicting results (Mastroeni et al., 2009; Bradley-Whitman and Lovell, 2013; Chouliaras et al., 2013; Condliffe et al., 2014; Coppieters et al., 2014; Lashley et al., 2015). Other candidate gene studies in AD postmortem brain tissues reported significant disease-associated differential DNAm at various genes implicated in AD pathogenesis, including *APP, GSK3B, MAPT, PP2AC, APOE, DNMT1, MTHFR, PGC-1α*, and *TREM2*, amongst others (Siegmund et al., 2007; Barrachina and Ferrer, 2009; Iwata et al., 2014; Silva et al., 2014; Chibnik et al., 2015; Foraker et al., 2015; Yu et al., 2015). Genome-wide DNAm profiling in superior temporal gyrus tissues using the Illumina 450K array found numerous differentially methylated regions, the majority of which showed AD-related increases in DNAm over controls, with notable overlap of hits to previous studies (De Jager et al., 2014; Lunnon et al., 2014; Watson et al., 2016).

In regard to PD, early candidate gene studies of DNAm in postmortem substantia nigra, putamen, and cortex samples revealed disease-related DNAm decreases at intronic CpGs of *SNCA* and *TNFα*, key genes associated with PD risk, although these associations have not been fully replicated or may be specific to distinct brain regions (Pieper et al., 2008; Jowaed et al., 2010; Matsumoto et al., 2010; de Boni et al., 2015). Interestingly, sequestration of nuclear DNMT1 by α-synuclein, encoded by *SNCA*, in brain samples from PD and Lewy body dementia patients has been attributed to global loss of DNAm at numerous genes, including *SNCA* itself, *SEPW1* and *PRKAR2A* (Desplats et al., 2011). On a genome-wide scale, DNAm profiling in postmortem brain samples has identified DNAm changes associated with PD risk variants at *PARK16*, *GPNMB*, and *STX1B* genes, signifying possible combined contribution of genetic and epigenetic variation on PD pathophysiology (International Parkinson's Disease Genomics Consortium (IPDGC), Wellcome Trust Case Control Consortium 2 (WTCCC2) , 2011).

Another DNAm study performed in putamen and cortex showed decreased DNAm and concomitant increase in expression of *CYP2E1* in PD brain over controls (Kaut et al., 2012). In addition, comparative analysis of DNAm profiles from matched brain and blood samples of PD patients and healthy controls revealed widespread differential DNAm at genes previously associated with PD pathology, including sites that exhibited high blood–brain DNAm concordance, suggesting that these associations may serve as potential blood-based PD biomarkers (Masliah et al., 2013).

Finally, DNA methylomes of prefrontal cortex samples from different neurogenerative disorders (i.e., AD, PD, and dementia with Lewy bodies) showed that the similar aberrant CpG methylation patterns across different disease entities targeted a defined gene set, signifying that common epigenomic alterations may contribute to distinct neurodegenerative states (Sanchez-Mut et al., 2016). Conversely, a recent study profiling mitochrondrial DNAm levels reported loss of mitochrondrial DNAm in the substantia nigra of PD patients over controls, while entorhinal cortex samples of AD patients exhibited increased mitochondrial DNAm over controls, signifying disease-specific alterations in the mitochondrial epigenome of neurons (Blanch et al., 2016). Overall, these results provide compelling evidence for the role of DNA modification variation in neurodegenerative diseases, particularly as it pertains to neuronal-specific pathology.

Mapping histone marks in neurodegenerative postmortem brain samples

Perturbation in chromatin structure and histone post-translational modification patterns is another source of

epigenomic variation that may contribute to neurodegenerative disease development and progression. Chromatin studies in HD postmortem brain show elevated levels of H3K9me3 in conjunction with increased histone H3K9-specific methyltransferase enzyme SETDB1 expression in neurons of HD striatum over controls (Ryu et al., 2006). Moreover, genome-wide mapping of H3K4me3 in neuronal chromatin from HD prefrontal cortex revealed 136 differentially enriched loci associated with genes implicated in neuronal development and neurodegeneration, including *GPR3, TMEM106B, PDIA6, HES4*, and *JAGGED2* (Bai et al., 2015). Interestingly, loss of H3K4me3 at the *HES4* promoter was associated with excessive DNAm and reduced binding of nuclear proteins to the methylated region, suggesting coordinated effects of histone post-translational modifications and DNAm on the HD neuronal epigenome (Bai et al., 2015).

In the AD postmortem brain, early antibody-based analyses of global phosphorylation and acetylation levels of histone H3 showed significant increases in H3 phosphorylation in AD frontal cortex tissues over controls but no significant differences were observed for global H3 acetylation levels (Rao et al., 2012). High-throughput proteomics analyses also revealed that H3K18ac and H3K23ac levels were significantly reduced in AD temporal lobe over age-matched controls (Zhang et al., 2012). Subsequent mass spectrometry-based quantification of multiple histone marks in AD frontal cortex reported significant decreases in methylation levels of H2B residue K108, H4 residue R55, as well as loss of acetylation near the N-terminus of H4; by contrast, ubiquitination of K120 on H2B showed a 91% increase in AD cases over controls (Anderson and Turko, 2015). A later study demonstrated that these dramatic changes in histone marks were proportional to changes in total histone protein, showing that increases in acetyl H3 and H4 levels were correlated with increases in total H3 and H4 levels in AD temporal gyrus, indicating that alterations in histone marks may be explained, in part, by alterations in histone protein homeostasis (Narayan et al., 2015).

Finally, in regard to PD, levels of histone acetylation were markedly higher in midbrain dopaminergic neurons of PD patients over controls, concordant with treatment-induced loss of HDACs, suggesting that PD environmental factors induce HDAC degradation and subsequent increases in histone acetylation of dopaminergic neurons (Park et al., 2016). A subsequent study performed in PD primary motor cortex tissue sought to delineate residue-specific differences in H3 acetylation levels, reporting elevated levels of H3K14ac and H3K18ac, decreases in H3K9ac, and no change in H3K23ac between PD cases and controls (Gebremedhin and Rademacher, 2016). Collectively, these results not only implicate histone post-translational modification variation in neurodegenerative disorders, but also showcase how chromatin alterations may be influenced by changes in cellular processes (i.e., autophagy, homoeostasis) or by environmental factors (i.e., pharmacologic treatments) in the context of neurodegenerative pathology.

Interrogation of noncoding RNA variation in postmortem neurodegenerative brain tissues

The role of ncRNAs in neurodegenerative processes has also garnered increasing interest across a number of degenerative brain disorders. For example, in HD pathogenesis, the long noncoding antisense transcript of the huntingtin gene (HTTAS), which negatively regulates *HTT* transcript levels depending on trinucleotide repeat length, exhibits reduced expression in HD frontal cortex over controls (Chung et al., 2011). Another study found that the HAR1 lncRNA was downregulated in the striatum of HD patients and that the HAR1 locus was targeted by the neural transcription factor, REST, which contributes to the widespread transcriptional suppression of neurons in HD pathogenesis (Johnson et al., 2010). More recent unbiased microarray-based analyses have reported alterations in numerous lncRNA species in human HD postmortem brain tissues over controls, including NEAT1, TUG1, MEG3, and DGCR5, amongst others (Johnson, 2012; Sunwoo et al., 2017). In addition to lncRNAs, a number of neuronal-specific microRNAs were dysregulated in HD brain tissue over controls in a manner that was consistent with HD-associated striatal depletion (Johnson et al., 2008; Hoss et al., 2015).

In the case of AD, dysregulation of numerous microRNA species, including miR-29, miR-34c, miR-106, miR-107, miR-125b, miR-146a, and miR-181, was observed in AD brain regions such as temporal-lobe neocortex and hippocampus over controls, in a manner that correlated with AD-related pathology (Hébert et al., 2008; Sethi and Lukiw, 2009; Nelson and Wang, 2010; Wang et al., 2011; Zovoilis et al., 2011; Schonrock et al., 2012). Interestingly, genes encoding a number of these miRNA species display differential DNA methylated in AD temporal cortex tissue over controls, highlighting potential interdependencies between different forms of epigenetic regulation in neurodegenerative disease progression (Villela et al., 2016).

Finally, aberrant miRNA expression patterns have also been linked to PD pathology in various brain regions. Specifically, disease-associated alterations have been reported for miR-205 and miR-34b/c, which regulate two familial PD risk genes *LRRK2* and *PARK2/7*, respectively, as well as miR-133b in postmortem PD brain (Kim et al., 2007; Miñones-Moyano et al., 2011; Cho et al., 2013). Taking a more comprehensive approach for miRNA discovery across multiple neurodegenerative

states, a recent study performed high-throughput small RNA sequencing to identify 99 novel miRNA candidates across prefrontal cortex samples of HD and PD patients as well as matched controls (Wake et al., 2016). Taken together, these findings highlight the complex regulatory roles of ncRNA species, particularly as they relate to pathologic neurodegeneration.

CHALLENGES AND CONSIDERATIONS RELATED TO EPIGENETIC STUDIES IN POSTMORTEM BRAIN

Despite substantial technologic advancements in the field, a number of key technical, methodologic, and analytical concerns still need to be addressed in regard to molecular analyses using human postmortem brain (McCullumsmith and Meador-Woodruff, 2011; Pidsley and Mill, 2011; Maze et al., 2014). Herein, we provide a provisional framework of issues and limitations to consider when performing epigenomic profiling in human postmortem brain samples. It is worth noting that general principles, practices, and recommendations for designing and interpreting epigenetic studies in human population cohorts have been extensively reviewed elsewhere and will not be covered in our discussion (Rakyan et al., 2011; Heijmans and Mill, 2012; Michels et al., 2013; Chadwick et al., 2015; Birney et al., 2016; Jones et al., 2017; Lappalainen and Greally, 2017). Thus, for the purposes of this chapter, we will specifically discuss challenges (both technical and biologic), as well as considerations relating to the use of postmortem brain samples in human epigenetic studies.

Challenges related to technical factors

Agonal state and pH

The presence of prolonged agonal status during death may profoundly affect the integrity and stability of biomolecules in the tissue obtained at autopsy. Agonal state refers to a condition characterized by shallow, labored breathing, medically known as agonal respiration, which often follows cardiogenic shock (Rea, 2005). Prolonged agonal state is associated with hypoxia, which acts to increase tissue lactate levels, thereby lowering pH in tissue (Hynd et al., 2003). Importantly, pH changes can affect RNA and protein integrity (Stan et al., 2006). In regard to specific epigenetic marks, both DNAm and histone methylation levels are relatively stable against alterations in pH (Huang et al., 2006; Ernst et al., 2008). These findings suggest that tissue pH may not directly affect methylation marks; however, its ability to confound other sources of epigenetic variation, either directly or indirectly through transcriptional changes, remains to be determined.

Postmortem interval

Postmortem interval (PMI) is defined as the time from death to tissue processing. A previous study showed that PMI, refrigeration interval, and temperature can significantly influence protein preservation in postmortem tissues, although different proteins exhibit variable vulnerability to these factors (Ferrer et al., 2008). A recent study examined the differential effects of PMI on the integrity of various biomolecules (RNA, miRNA, histone post-translational modifications, and proteins) using rat postmortem brain samples (Nagy et al., 2015). The study found that miRNAs were largely resistant to lengthy PMIs (i.e., 96 hours), whereas histone post-translational modifications were stable to thresholds between 72 and 96 hours and histone proteins lasted up to 72 hours. Interestingly, histone H3 methylation was quite stable across all tested time points (up to 96 hours), while H3 acetylation marks exhibited more variability, indicating that histone acetyl groups may be less stable than histone methyl marks (Nagy et al., 2015). Together, these results suggest that the effects of PMI may differ depending on the biomolecule being measured. Further investigation is needed to discern how PMI affects various epigenetic marks, including DNAm, in human postmortem brain tissues.

Tissue dissection and processing

Disparate results in postmortem epigenetic studies using different brain collections may be attributable to differences in dissection protocols, tissue processing, and/or nomenclature. For example, tissue from the dorsolateral prefrontal cortex could originate from Brodmann area 9 or 46, and regions such as the hippocampus could exhibit marked functional and cellular differences depending on the position of sampling in the anterior/posterior axis (Webster, 2006). In terms of tissue processing, the best mode of preservation for quality nucleic acid extraction is rapid freezing of fresh tissue and storage at –80°C (Ferrer et al., 2008). Other procedures of tissue preservation such as formalin fixation and paraffin-embedding are also widely used, but can compromise the quantity and quality of DNA, RNA, and protein (Ferrer et al., 2008). Additional factors such as tissue autolysis and numerous freeze–thaw cycles can further diminish sample integrity. A former study reported that histone methylation was more readily maintained across a wide range of autolysis times and tissue pH than histone acetylation, further highlighting the differential sensitivity of distinct histone post-translational modifications to technical confounds (Huang et al., 2006). This study also showed that the bulk of nucleosomal DNA remained attached to histones during the first 30 hours after death and that

immunoprecipitation with antibodies against methylated histones was at least 10-fold more effective in unfixed micrococcal nuclease-digested samples in comparison to extracts prepared by fixation and sonication (Huang et al., 2006). This potential for technical variability has motivated the development of standardized ChIP protocols to assess histone post-translational modifications in postmortem brain chromatin (Huang et al., 2006; Jiang et al., 2008; Akbarian and Huang, 2009; Kundakovic et al., 2017). At present, standardized protocols for the analysis of ncRNA and DNAm in postmortem human brain tissues have not been established, although it is thought that DNAm is relatively stable during tissue processing and long-term storage, thereby serving as a reliable measure of epigenetic variation in postmortem brain samples (Pidsley and Mill, 2011).

Challenges related to biologic factors

Cellular specificity of epigenetic marks

Brain tissues are highly heterogeneous and comprise many different cell types, including neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor cells, microglia, and vascular cells (Darmanis et al., 2015). Due to their role in establishing and maintaining cellular identity, neuroepigenetic measures exhibit substantial cell type specificity. Interindividual differences in cell composition can often confound epigenetic analyses by masking true associations or altering epigenetic signatures to give rise to spurious associations (Rakyan et al., 2011; Heijmans and Mill, 2012; Michels et al., 2013; Chadwick et al., 2015; Birney et al., 2016; Jones et al., 2017; Lappalainen and Greally, 2017). This is particularly important in the context of epigenetic studies in postmortem neurodegenerative brain samples, since loss of specific neurons in distinct brain regions, often in conjunction with increased proliferation of glial cells (gliosis), is a salient feature of neurodegenerative pathology (Kwon et al., 2016).

To address cellular heterogeneity in epigenetic analyses of postmortem brains, two broad approaches can be employed: (1) cell type adjustment methods of epigenetic measures from bulk tissue; and (2) isolation of target cell(s) for epigenetic interrogation. In the first approach, which has been widely used in the context of DNAm studies of human brain, cell type proportions can be estimated based on underlying reference profiles from isolated cells (neuronal vs. nonneuronal populations) or "reference-free" methods can be applied which correct for the effects of cell composition without actually predicting cell proportions (Guintivano et al., 2013; Montaño et al., 2013; Houseman et al., 2014; Zou et al., 2014). In the second approach, which has been used for chromatin studies in brain tissues, neuronal nuclei are selectively tagged for subsequent fluorescent-activated cell sorting and ChIP-based profiling of specific histone marks (Jiang et al., 2008; Kundakovic et al., 2017). Alternatively, laser capture microdissection can be used to isolate defined cell populations from specific brain regions, although this method is quite expensive and labor-intensive and has not yet been used in epigenetic analyses in human brain tissue (Suarez-Quian et al., 1999). Regardless of the approach, controlling for cellular heterogeneity in epigenetic studies of postmortem brain tissues is an integral step in uncovering true epigenetic associations to neurobiologic phenotypes.

Sample selection

A number of individual-specific biologic factors can contribute to variability in epigenetic signals, including diagnostic or phenotypic heterogeneity, prior medication exposure, age, sex, and ethnicity. As these factors can potentially confound neuroepigenetic findings, they should be carefully considered and described in the selection of postmortem brain samples for epigenetic study cohorts.

In terms of diagnostic or phenotypic heterogeneity, many of the neurobiologic phenotypes of interest in human epigenetic studies exist as syndromes which display a wide range of clinical symptoms and varying degrees of each diagnostic characteristic. To reduce such phenotypic variability, well-characterized endophenotypes are being developed to clarify descriptive diagnostic criteria into more stable phenotypes with clear genetic linkages (Hasler et al., 2004; Hazlett et al., 2007). In addition, the inclusion of an illness comparison group can help discern if an epigenetic association is linked to a particular disorder, as opposed to a generalized effect of disease in the brain (Dorph-Petersen et al., 2009; Deming et al., 2016). For example, a previous study delineated shared versus distinct epigenetic signatures between the neurodegenerative disease modalities in postmortem brain tissues from individuals with AD, dementia with Lewy bodies, PD, and Alzheimer-like neurodegenerative profile associated with Down syndrome (Sanchez-Mut et al., 2016). These approaches, along with well-defined inclusion and exclusion criteria, can help limit phenotypic heterogeneity in epigenetic study cohorts.

Medication history is another factor that can confound epigenetic findings from postmortem brain. For instance, chronic administration of levodopa, a psychoactive drug routinely used for PD treatment, has been associated with dose-dependent increases in *SNCA* DNAm and marked H4 deacetylation (Nicholas et al., 2008; Schmitt et al., 2015). However, it is unclear whether these epigenetic changes are associated with exposure to the drug itself or secondary effects induced by drug treatment (i.e., levodopa-induced dyskinesia).

Another major contributor to neuroepigenetic variability is age, which has been widely assessed in the context of DNAm variation (reviewed in Jones et al., 2015). Specifically, in normal human brain tissues, aging is associated with a global loss of genome-wide DNAm, in conjunction with a gain of DNAm at specific loci (Lister et al., 2013). Another measure that is also relevant in this context is epigenetic age, which is hypothesized to represent an estimate of biologic aging and may vary between samples (Horvath, 2013). Such analyses in postmortem brain have been made possible by the development of a pan-tissue "epigenetic clock" that calculates the epigenetic age of a sample based on DNAm levels at 353 CpG sites (Horvath, 2013). Recent studies performed in postmortem brain tissues from HD and AD patients showed accelerated epigenetic aging in HD brain, particularly in cortical tissues, as well as an association between epigenetic age and AD-related cognitive decline (Levine et al., 2015; Horvath et al., 2016). These findings suggest potential links between biologic aging, epigenetic alterations, and neurobiologic disease states in postmortem brain tissues. As such, accounting for age-related differences in epigenetic measures is an imperative consideration in postmortem epigenetic studies of human brains.

Sex differences can also influence epigenetic variation (McCarthy et al., 2017). In addition to the Y chromosome, one of the principal sex-specific differences is the inactivation of one of the two X chromosomes in females, which is coordinated by numerous epigenetic mechanisms, including DNAm, chromatin remodeling, and ncRNAs (Avner and Heard, 2001). Sex-specific epigenetic differences may also occur on autosomes, particularly in the context of disease, as demonstrated by a previous study which reported sex-specific differences in psychosis-associated DNAm signatures from schizophrenic and bipolar disorder frontal cortex tissues (Mill et al., 2008). Thus, balancing sex between comparison groups or accounting for it in subsequent analyses is an important aspect in controlling for sex-specific differences in human epigenetic studies.

Finally, ethnicity differences may influence epigenetic patterns in part through population-specific genetic influences on epigenetic marks as well as through culturally associated differences in lifestyle, diet, or habitat (Fraser et al., 2012; Fagny et al., 2015; Galanter et al., 2017; Jones et al., 2017). For example, genetic variation and DNAm are closely linked, particularly in the context of mQTLs, which can be tissue- and age-specific but also vary across different populations and ethnic groups (Fraser et al., 2012; Gutierrez-Arcelus et al., 2013; Banovich et al., 2014; Teh et al., 2014; Hannon et al., 2016; Van Dongen et al., 2016). In addition, ethnicity-driven patterns in epigenetic variation may arise from cultural and environmental commonalities. For instance, a recent study in two African populations showed that DNAm alterations associated with historically different lifestyles were related to developmental processes, while DNAm changes associated with current habitat implicated cellular and immune functions (Fagny et al., 2015). Overall, these observations provide compelling evidence that ethnicity differences are associated with epigenetic patterns either directly through ancestry-related genetic variation or indirectly through culturally dependent associations. As such, accounting for population stratification in cohort group comparisons is imperative in the context of human epigenetic studies.

Taken together, these individual-specific biologic factors can greatly confound epigenetic analyses, leading to spurious findings or masking true associations. Therefore, great care should be taken to identify and control for these biologic covariates during sample collection and analysis.

Considerations for epigenetic studies in postmortem human brain

Central versus peripheral tissues

In addition to understanding human epigenetic variation in target brain tissues, there is increasing interest in studying neuroepigenetic alterations related to biologic phenotypes in peripheral tissues. This has been largely motivated by the fact that postmortem human brain tissues are relatively scarce in comparison to more readily accessible tissues that may be used as surrogates, such as blood, saliva, or buccal epithelial cells. Furthermore, studies in postmortem human brain samples can only be performed retrospectively and do not allow for longitudinal tracking of epigenetic changes associated with phenotypes of interest. As such, readily accessible peripheral tissues have become increasingly attractive for the exploration of epigenetic biomarkers, which may aid in early disease detection and monitoring (Bock, 2009).

A key prerequisite in the use of surrogate tissues for epigenetic biomarker discovery of brain-related phenotypes is that epigenetic patterns from peripheral tissues should reflect those in the brain. Recent studies have sought to address this by exploring DNAm concordance between matched human blood and brain tissues, reporting mixed patterns in which some DNAm sites are highly concordant between tissues while others are discordant (Davies et al., 2012; Farré et al., 2015; Hannon et al., 2015; Edgar et al., 2017). Importantly, various publicly available resources have been created to provide DNAm profiles of matched blood and brain tissues at individual CpGs and allow researchers to discern if DNAm associations in blood are similar in brain (Hannon et al., 2015; Edgar et al., 2017). This approach has been demonstrated

in previous studies of psychosis in discordant monozygotic twins to show that blood-based DNAm findings could be replicated in postmortem brain tissues (Sugawara et al., 2011; Fisher et al., 2015). Parallel efforts for other peripheral tissues and epigenetic marks will greatly promote epigenetic biomarker discovery in the context of human neurobiologic phenotypes.

Data sharing and integration

Given the difficulty in obtaining high-quality postmortem human brain tissue, the development of publicly available resources and repositories for sharing molecular data from postmortem human brain samples have become a high priority. Large-scale consortiums, such as the National Institutes of Health Roadmap Epigenomics Consortium and PsychENCODE, have initiated large-scale projects to generate human reference epigenomes, profiling numerous histone post-translational modifications, chromatin accessibility, DNAm, and RNA expression across multiple different brain regions and brain-specific cell types (Akbarian et al., 2015; Kundaje et al., 2015). These efforts not only allow for the development of tools and resources for the larger scientific community to fuel replication efforts of epigenetic associations, but also facilitate multidisciplinary collaborations to intersect expertise and resources.

Notably, these types of data-sharing initiatives are not only crucial for the reproducibility of neuroepigenetic findings, but are also important resources for integrative analyses aimed at combining molecular data types, including different epigenetic marks, genetic variation, RNA expression, protein levels, and chromosomal conformation (Ng et al., 2017). For example, H3K4me3 distribution patterns in prefrontal cortex neurons show higher-order chromatin structures in direct contact with multiple H3K4me3 peaks at key neurodevelopmental disease loci, suggesting coordinated regulation by histone marks with higher-level chromatin features (Shulha et al., 2012b). Such integrative analyses are gaining traction due to their potential to provide insights into robust biologic variation in the human brain, particularly as they pertain to neurobiologic disease states.

CONCLUSIONS AND FUTURE DIRECTIONS

Advancements in methodologies to assess epigenetic variation have galvanized efforts to characterize molecular profiles in human postmortem brain tissues. Specifically, postmortem epigenomic profiles, constituting DNA modifications, chromatin structure, and ncRNA expression, have been mapped across a broad range of human brain tissues and developmental contexts, shedding new light on our understanding of epigenetic regulation in neural function and plasticity across the life course. Importantly, postmortem interrogation of brain-specific epigenetic alterations associated with neurobiologic disease phenotypes, including neurodevelopmental, psychiatric, and neurodegenerative disorders, has provided compelling insight into the gene-regulatory architecture underlying disease susceptibility and pathogenesis. However, the use of postmortem brain tissues for molecular analyses warrants careful consideration of key technical and biologic factors, which may confound epigenetic analyses. These challenges may be mitigated by the establishment of standardized protocols to archive high-quality, well-characterized postmortem tissues in brain biobanks, along with vetted procedures to effectively measure epigenetic marks in postmortem brain, thereby allowing for more systematic assessments of human epigenomic variation in postmortem brain tissue.

The field of neuroepigenetics is rapidly expanding to provide novel opportunities to characterize molecular features of brain-related function and pathology using studies from postmortem brain tissues. The development of new methodologies, such as single-cell epigenomic profiling, has the potential to transform our understanding of neuronal subtype diversity in heterogeneous brain tissues, as well as more deeply interrogate the epigenetic regulatory landscape in individual cell types from neural tissues (Schwartzman and Tanay, 2015; Clark et al., 2016). These techniques have yet to be applied in the context of human postmortem brain tissues, but reflect promising new avenues for investigating the neuronal epigenome.

Finally, it is worth mentioning that, by nature, epigenetic findings from analyses in postmortem brain can only be descriptive and correlational, as it is not possible to manipulate or perturb tissues that have been retrospectively obtained. Consequently, hypothesized causal mechanisms must be tested using in vitro and in vivo experimentation, although postmortem data can be used to identify priorities in such experimental work. As such, future efforts will likely focus on deriving mechanistic insights by testing associations obtained from postmortem brain analyses in cell lines and animal models to gain a clearer understanding of the causal molecular relationships that underlie human brain function and pathogenesis.

ACKNOWLEDGMENTS

We would like to thank Drs. Sarah Moore and Meaghan Jones for their guidance and feedback. MSK is a Senior Fellow of the Canadian Institute for Advanced Research and the Canada Research Chair in Social Epigenetics. AAL is supported by a Developmental Neurosciences Research Training Award from Brain Canada and NeuroDevNet.

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