Current Topics in Behavioral Neurosciences 42

Elisabeth B. Binder
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Behavioral Neurogenomics

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Behavioral Neurogenomics

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Preface

Understanding the interaction of genotype, environment, and phenotype is a central challenge in decoding normative and disease-associated behavior. We are currently at a critical intersection of increasingly larger, population-wide human studies and rapid technological advances, particularly in the fields of neurobiology, genomics, and digital phenotyping. Efforts combining high-throughput genomic technologies with global collaborative clinical studies have been rewarded, for example, by a growing number of robust, replicable genetic associations from genome-wide association studies of psychiatric disorders. Moreover, human and animal model studies provide converging evidence for the profound effects of environmental factors on long-lasting behavioral changes, which are likely mediated by epigenetic factors. Recent technological development in single-cell technologies, human in vitro cellular models, and functional genomics now provide the opportunity to understand how genetic and epigenetic factors influence gene and cell function and by this shape behavior and disease risk. Combining these new technologies with robust clinical studies carries the promise of a better understanding of the etiology, prevention, and treatment of psychiatric disorders.

This book illustrates how these new developments have recently furthered our understanding in the area of *Behavioral Neurogenomics*. In the first chapter, Divya Mehta and Darina Czamara examine the progress in genome-wide association studies, which arguably provide unprecedented insight into the complex genetic architecture of behavioral traits and psychiatric disorders. At the same time, these studies fall short in providing mechanistic insight into genetic moderation of certain behaviors or disease risk. In part this is due to the limited understanding of genome organization and function. John Fullard, Samir Rahman, and Panos Roussos focus in the second chapter on cis-regulatory elements in our genome and how genetic variation identified through large-scale studies may influence non-coding, regulatory genomic features. Fullard and colleagues provide an example of how association study findings may be utilized to investigate the molecular mechanisms of genetically driven phenotypes. However, besides behavioral variance/variation as a function of genotype, environmental factors can have long-lasting effects on behavior

and disease risk. Two chapters, one by Rustad, Papale, and Alisch and the other by Merrill, Gladish, and Kobor, focus on mechanisms of how environmental factors may change molecular trajectories that eventually have an impact on behavior. While Rustad and colleagues focus on the dynamic function of DNA methylation and hydroxymethylation in the brain, Merill, Gladish, and Kobor focus on how the social environment, in particular in early life, shapes epigenetic modifications with long-lasting effects across the life span. The chapter by Fischer expands the view on epigenetic modifications and behavior specifically focusing on the role of histone modifications in learning and memory formation. Importantly, Fischer discusses the enzymatic machinery involved in histone biology and points to potential therapeutic opportunities to treat brain diseases. In the next chapter, Kaindl and Winner highlight the opportunities that arise with human *in vitro* cellular models as animal models currently cannot model the complex genetic heterogeneity of human behavior. Murphy and Singewald then return to molecular mechanisms underlying behavior. Their focus on microRNAs, a species of non-coding RNAs strongly expressed in the brain, highlights the importance of regulatory RNAs expressed in central hubs of the anxiety neurocircuitry modulating behavior in animal models. Furthermore, they link microRNA expression with known pathways regulating neurotransmitter release and signaling, synaptic plasticity, and stress-hormone axis function. Guffanti, Bartlett, DeCrescenzo, Macciardi, and Hunter explore the far less known role of transposable elements. The depiction of transposable elements as junk DNA seems shortsighted and data reviewed by Guffanti and colleagues provide interesting insights into the possible function of these elements in the brain influencing behavior and risk for disease. Finally, Vassoler, Toorie, and Byrnes review evidence for inter- and transgenerational effects of environmental cues. Behavioral phenotypes associated with inter-, multi-, and transgenerational effects following a variety of parental exposures are intriguing and may have an important role in shaping disease risk. Yet, the molecular substrate linked to the transmission of these cues remains elusive with multiple studies pointing towards DNA methylation or small RNA signaling mechanisms.

The chapters in this volume of *Current Topics in Behavioral Neurogenomics* thus provide a broad overview of the dynamic field of *Behavioral Neurogenomics*. They are written for both basic scientists and research-oriented clinicians and provide an in-depth introduction into the various areas of research. We believe that additional technological developments in basic and clinical neurogenetics will further accelerate our understanding of how genetics, environmental factors, and the resulting phenotypes interact to shape human behavior in health and disease. Knowledge of the underlying machinery driving human behavior will eventually influence preventive, diagnostic, and therapeutic strategies for complex psychiatric disorders.

Munich, Germany — Elisabeth B. Binder
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GWAS of Behavioral Traits

Divya Mehta and Darina Czamara

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Abstract Over the past decade, genome-wide association studies (GWAS) have evolved into a powerful tool to investigate genetic risk factors for human diseases via a hypothesis-free scan of the genome. The success of GWAS for psychiatric disorders and behavioral traits have been somewhat mixed, partly owing to the complexity and heterogeneity of these traits. Significant progress has been made in the last few years in the development and implementation of complex statistical

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methods and algorithms incorporating GWAS. Such advanced statistical methods applied to GWAS hits in combination with incorporation of different layers of genomics data have catapulted the search for novel genes for behavioral traits and improved our understanding of the complex polygenic architecture of these traits.

This chapter will give a brief overview on GWAS and statistical methods currently used in GWAS. The chapter will focus on reviewing the current literature and highlight some of the most important GWAS on psychiatric and other behavioral traits and will conclude with a discussion on future directions.

Keywords Genome-wide association studies · Heritability · Psychiatric disorders

1 Introduction to Genome-Wide Association Studies (GWAS)

In genome-wide association studies (GWAS), association analysis between a categorical or quantitative trait and genome-wide single-nucleotide polymorphisms (SNPs) is performed. The number of single-nucleotide polymorphisms (SNPs) involved in disease susceptibility discovered in GWAS has increased considerably throughout the past decades (Visscher et al. 2012). Two main reasons drove this high increase in GWAS: the fast progressing development of new technologies and the fact that SNP genotyping on whole-genome level became not only available but also cheaper and quicker. Today, millions of SNPs can be genotyped using high-throughput methods. Furthermore, Risch and Merikangas (1996) showed that association analysis has greater power to detect susceptibility loci as compared to linkage analysis if the loci exert only a small effect on the disease. In the 1990s, linkage studies, which are familial studies assessing if markers are inherited to affected offspring more often than would be expected by chance, were commonly performed. However, the linkage method performs best if rare mutations with large effects are present, but as the genetic basis of psychiatric and behavioral disorders is rather complex (Smoller 2014), association studies are an ideal tool. GWAS in particular are suited for this purpose because they are hypothesis-free as SNPs throughout the whole genome are analyzed. This is in contrast to candidate gene studies that focus on specific genetic regions identified in previous studies or of interest because of their biological function.

In the first GWAS, susceptibility genes for several complex traits including type I diabetes (Wellcome Trust Case Control C 2007), type II diabetes (Sladek et al. 2007), and breast cancer (Easton et al. 2007) were identified. Nevertheless, for other complex diseases, this approach was not very successful, with only few genome-wide significant loci, or borderline associations were observed.

Above a certain "inflection point" (a critical sample size), the number of observed significant associations increases linearly with the sample size (Levinson et al. 2014). This was described, for example, for lipids and blood pressure which present

with linear or even more rapid increase in findings for sample sizes above this critical threshold (Panagiotou et al. 2013). Based on this, it is very likely that complex diseases are predicted by a polygenic model where a high number of SNPs, each one only exhibiting a small effect size, are involved in disease susceptibility. A consequence of this is that large sample sizes of several thousand individuals are necessary to detect such small effects and indeed, the larger the sample sizes got, the more association hits were found (Visscher et al. 2012; Levinson et al. 2014).

To reach sample sizes of thousands of individuals, a large number of national and international consortia have been established, which combine datasets of individual studies in meta- or mega-analyses. One of the first was the Wellcome Trust Case Control Consortium (WTCCC) in 2005 which studied diabetes, bipolar disorder, and Crohn's disease, among other disorders (Wellcome Trust Case Control C 2007). The Psychiatric Genomics Consortium (PGC) is one of the largest consortia today including over 900,000 samples across several psychiatric disorders such as schizophrenia, bipolar disorder, major depression, or ADHD (Sullivan 2010).

2 Methods for GWAS

2.1 Simple Analysis of Single Markers

In a genome-wide association study, the association between a phenotype of interest and SNPs spread across the whole genome is tested. Different genotyping arrays covering up to 2,300,000 SNPs are available. After array processing, genotypes for each sample are called based on their signal intensities. This step is followed by quality control which removes SNPs as well as individuals with low performance. Usually, SNPs with low call rates, with low minor allele frequency, and with significant deviation from Hardy-Weinberg equilibrium as well as individuals with low call rates and large deviations in mean heterozygosity with respect to the whole sample are excluded. The remaining SNPs and samples are then used for further statistical analysis.

Not all SNPs are covered on genotyping arrays; however, based on the structure of the human genome in linkage disequilibrium (LD) blocks, missing genotypes can be predicted to a certain extent. Large samples from different ethnicities that have been sequenced such as the HapMap (International HapMap C 2003) and the 1000 Genomes projects (Genomes Project et al. 2010) are available for imputation. Based on the LD pattern in these reference samples, missing genotypes can be imputed in the study samples (Marchini et al. 2007), and the imputation algorithms work quite accurately (Howie et al. 2009). After imputation and quality control, association analysis is performed.

Very often affected individuals, for example, depression cases, are compared to healthy control subjects with regard to significant differences in allele frequencies. This comparison can be performed with logistic regression where also possible confounding variables, such as age, gender, or ethnicity, can be used as covariates

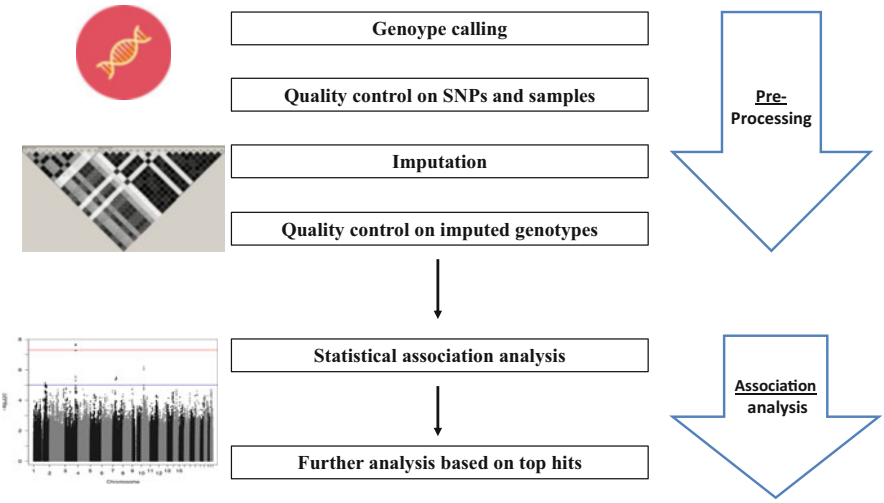

Fig. 1 GWAs workflow

in the analysis. In the GWAS summary statistics results, for each SNP the *p*-value, odds ratio (OR), standard error, and the reference allele are indicated.

If the phenotype of interest is a quantitative trait, a depression scale, for example, linear regression, is used which reports the effect size as the mean increase/decrease of the phenotype when a further copy of the reference allele is present; this analysis also allows the addition of covariates. On a genome-wide level, both logistic and linear regression can be easily performed using freely available analysis tools such as PLINK (Chang et al. 2015; Purcell et al. 2007).

Based on the assumption that one million independent SNPs are present in the European human genome, *p*-values below the threshold of 5×10^{-08} are considered as genome-wide significant (Pe'er et al. 2008). In the next steps, top hits (SNPs) are more closely investigated with regard to their genomic position, and further analyses, such as pathway analysis, are performed. Figure 1 summarizes the preprocessing and analysis pipeline of GWAS.

2.2 *Using GWAS Data to Investigate the Complex Genetic Architecture of Psychiatric Disorders: Heritability, Genetic Correlation, and Polygenic Risk Scores*

Increased risk of psychiatric disorders among relatives of affected individuals points toward a genetic contribution for these disorders, and this has led to a plethora of genome-wide association studies (GWAS) conducted for psychiatric disorders. Nevertheless, so far, GWAS have identified very few genome-wide significant

SNPs for psychiatric disorders, and the identified SNPs only explain a small proportion of variance of the phenotype. These findings suggested that newer methods were needed to account for the polygenic genetic architecture of psychiatric disorders. More recent methods demonstrated that variants associated with a particular behavioral trait can also be aggregated into a single empirical polygenic genomic risk score for each individual (Ruderfer et al. 2014). The polygenic genomic risk score method (GPRS) takes statistically independent risk alleles and their effect sizes from a GWAS (discovery or training set) and, after LD pruning, uses these to generate a combined genomic profile risk score for each individual in an independent target sample (Wray et al. 2014). A GPRS is defined as the sum of the count of risk alleles weighted by the effect size in the discovery sample (Iyegbe et al. 2014). Different inclusion thresholds for selection of SNPs based on the GWAS significance can be used to build GPRS via software tools such as PLINK (Chang et al. 2015; Purcell et al. 2007) and PRSice (Euesden et al. 2015). An advantage of this method is that the statistical power is dependent on the training set; hence, a larger training data can be used for risk prediction in smaller target samples. The GPRS method has been widely applied both within and across psychiatric disorders analyses (Cross-Disorder Group of the Psychiatric Genomics C 2013; International Schizophrenia C et al. 2009).

Genome-wide genotype data can also be used to estimate additive heritability attributable to common genetic variation, known as "SNP heritability" (h^2_{SNP}) (Lee et al. 2011a; Yang et al. 2010). Estimation of the variance explained by all SNPs (h^2_{SNP}) or the genetic similarity between unrelated individuals in the study is performed using methods called GREML (genome-based restricted maximum likelihood) (Benjamin et al. 2012). The GREML-based method GCTA (genome-wide complex trait analysis) can be used to assess the degree of genetic relatedness between individuals by building a genetic relationship matrix, based on the assumption that cases are genetically more similar to each other than to controls (Yang et al. 2011). The genetic relationship matrix is then correlated with dichotomous or quantitative phenotypes (Lee et al. 2011a; Yang et al. 2010, 2011; Speed et al. 2012). An extension of GCTA is the estimation of genetic correlation (rg_{SNP}) explained by GWAS SNPs between two disorders (Lee et al. 2012a), with a positive correlation indicating that the cases of one disorder show higher genetic similarity to the cases of the other disorder than to their own controls. This method can also be used to calculate SNP-based co-heritability across pairs of disorders. Both GPRS and GREML methods can be applied simultaneously to datasets to provide further evidence for shared polygenic relationships between traits. A limitation of these methods is that they are computationally intensive and require individual-level genotype data.

The limitations of the conventional methods sparked the development of a newer method termed cross-trait linkage disequilibrium (LD) score regression that enables estimation of genetic correlation using only summary statistics from GWAS (Bulik-Sullivan et al. 2015a). This method does not require individual genotypes, genome-wide significant SNPs, or LD pruning and is computationally fast. The LD score regression approach functions under the assumption that if a trait is genetically

influenced, then variants in high LD would tag causal variants and be more significantly associated with the trait and hence have higher summary statistics as compared to variants with low LD. This method is flexible and can be adapted to estimate SNP heritability (Bulik-Sullivan et al. 2015a), partition SNP heritability by functional categories (Finucane et al. 2015), and can be used to estimate genetic correlation between different psychiatric disorders (Bulik-Sullivan et al. 2015b). Based on this method, the LD Hub web interface was built, which is a centralized database (http://ldsc.broadinstitute.org/) that allows calculation of heritability and genetic correlation via LD score regression analysis of user GWAS data against summary-level GWAS results for 173 diseases/traits (Zheng et al. 2017).

The above described methods complement each other and capitalize on GWAS data to gain further insights into the genetic architecture of psychiatric disorders.

2.3 Methods for Identification of Causal Genes from GWAS via Gene Enrichment, Network Analysis, and Other Functional Data

GWAS have been implemented for complex traits such as psychiatric disorders; however, identifying new risk loci and interpreting the results have been more challenging than anticipated. LD between SNPs forms the major backbone of GWAS, but it also makes it difficult to disentangle causal variants from nonfunctional variants co-occurring in large LD blocks. As a result, genetic variants and genes identified in GWAS are generally aggregated via pathway and network-based methods according to shared biology or function to identify likely causal genes within GWAS-implicated loci (Kao et al. 2017). Figure 2 shows different paths from identification of GWAS SNPs to unraveling risk genes for psychiatric disorders.

Gene set analysis allows to prioritize loci where multiple SNPs or genes within particular biological processes or molecular function groups show evidence of association. A widely used gene set analysis method is gene set enrichment analysis (GSEA) that evaluates whether members of a particular gene set tend to be more significantly correlated with the phenotype than expected by chance alone (Subramanian et al. 2005). Gene set analysis methods can bypass stringent multiple-testing corrections needed for analysis of individual signals and concentrate on biologically distinct sets of genes or processes, hence increasing the power of the study. Numerous public databases have pre-available gene sets that can be used for gene set and pathway analyses, including the Gene Ontology (Ashburner et al. 2000), WikiPathways (Kutmon et al. 2016), the Pathway Commons database (Cerami et al. 2011), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000), and DAVID (da Huang et al. 2009; Huang et al. 2007), among others. Other pathway-based GWAS analysis tools include INRICH (INterval enRICHment analysis) that tests for enriched association signals of

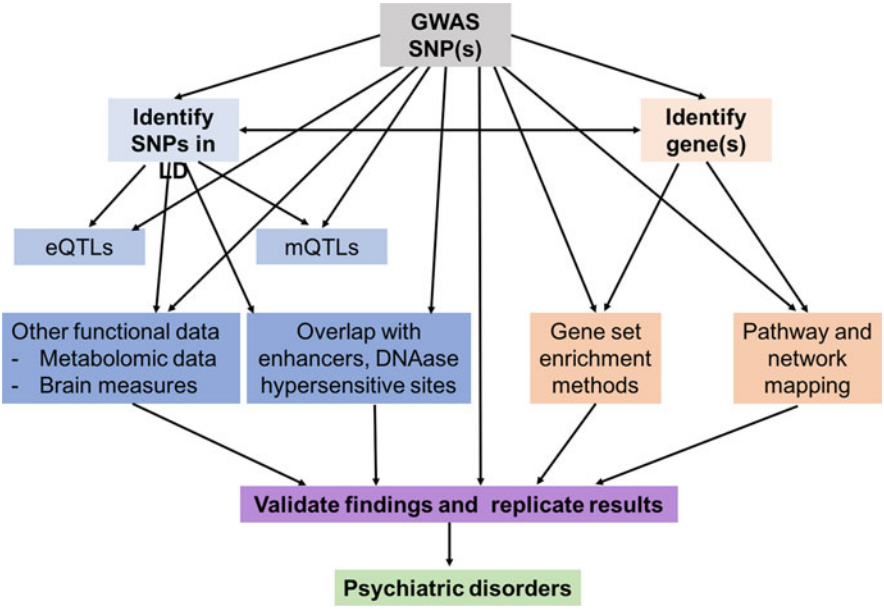

Fig. 2 From GWAS to psychiatric disorder

predefined gene sets across independent genomic intervals (Lee et al. 2012b) and Meta-Analysis Gene Set Enrichment of Variant or MAGENTA (Segre et al. 2010) that tests for enrichment of genetic associations in predefined biological processes or sets of functionally related genes. Enriched gene sets might offer deeper insight into disease mechanisms and biology of disease.

Different causal genes for the same phenotypic trait often directly interact or interact via common interaction partners, by the principle of "guilt by association"; hence, network-based approaches can also be useful to pinpoint putative causal gene(s) from LD blocks by finding genes that are nearby or related in a network to other known causal genes. Biological networks, such as protein-protein interaction (PPI) networks, can be used to define gene sets by selecting genes that interact at the protein level and plotting direct PPI neighbors and the interactions between these proteins to visualize large protein networks (Cowley et al. 2012).

Finally, using quantitative information such as gene expression or DNA methylation data can also reveal GWAS variants that are more likely to have consequences on gene activity or function. GWAS variants associated with gene expression are referred to as expression quantitative trait loci (eQTL) or expression SNPs (eSNPs). It has been demonstrated that complex trait-associated SNPs identified by GWAS are more likely to be eQTLs, confirming that genetic risk factors for complex traits will often affect phenotype by altering the amount or timing of protein production (Nicolae et al. 2010). Harnessing gene expression information can therefore enhance discovery of trait-associated SNPs and help us to gain a better understanding of the biology underlying complex traits.

Gene set enrichment and network-based methods described above are limited by the existing knowledge of the biological pathways and hence can have limited coverage and might miss interactions, reducing the sensitivity of these analyses. Despite this, such methods provide a further step toward the prioritizing candidate genes or causal variants for further experimental validation. Extensive analysis and combination of computational and experimental approaches will yield mechanistic insights into the molecular process by which a genetic variant(s) influence psychiatric disorders.

3 GWAS on Psychiatric Disorders, Behavioral, and Personality Traits

In the following paragraphs, we give a brief outline on GWAS findings for behavioral traits. In reviewing existing literature, we put the focus on the most important findings and/or the largest published studies; this review does not claim completeness.

3.1 Schizophrenia

Schizophrenia is a severe, chronic mental health disorder which is characterized by a variety of symptoms, including delusions, hallucinations, disorganized speech or behavior, and impaired cognitive abilities (Patel et al. 2014). It presents with a lifetime risk of 1% and is highly heritable with heritability estimates of up to 80% (Cardno and Gottesman 2000; Sullivan et al. 2003), stressing the importance of genetic variants in the etiology of schizophrenia. The first GWAS on schizophrenia (Mah et al. 2006; Lencz et al. 2007; Sullivan et al. 2008; O'Donovan et al. 2008; Shifman et al. 2008; Kirov et al. 2009; Need et al. 2009) were underpowered to find any genome-wide significant hits (Bergen and Petryshen 2012). The first consortia papers were published by the International Schizophrenia Consortium (ISC) (International Schizophrenia C et al. 2009), Molecular Genetics of Schizophrenia (MGS) (Shi et al. 2009), and SGENE (Stefansson et al. 2009). They reported the first genome-wide significant findings in *MHC*, *TCF4*, and *NRGN*. Apart from other GWAS with nonsignificant findings (Chen et al. 2011; Athanasiu et al. 2010), Williams et al. (2011) added *ZNF804A* to the list of genome-wide significant hits, while Steinberg et al. (2011) replicated the *MHC* and *TCF4* associations and additionally reported genome-wide significant variants in VRK2 to be associated with schizophrenia.

The sample size increased to over 9,000 cases and over 12,000 controls in the first Psychiatric Genomics Consortium (PGC) schizophrenia paper (Schizophrenia Psychiatric Genome-Wide Association Study C 2011), where 7 genome-wide

significant hits including 5 new loci (*MIR137*, *PCGEM1*, *CSMD1*, *MMP16*, and *CNNM2/NT5C2*) were reported.

Interestingly, the microRNA *MIR137* and its gene targets (*TCF4*, *CACNA1C*, *CSMD1*, and *C10orf26*) were genome-wide significant in either the schizophrenia or the combined schizophrenia-bipolar disorder PGC analyses (Schizophrenia Psychiatric Genome-Wide Association Study C 2011), suggesting a shared biological pathway in schizophrenia and bipolar disorder.

Also, GWAS on non-Western populations have been published (Ikeda et al. 2011; Yue et al. 2011; Shi et al. 2011a): Yue et al. (2011) reported a novel association at 11p11.2 and could replicate associations in the *MHC* region, while Shi et al. (2011a) found two new loci at 8p12 and 1q24.2.

Combining the PGC cohorts and a Swedish sample, Ripke et al. (2013) showed that many independent, mostly common SNPs collectively accounted for at least 32% of the variance in the liability to schizophrenia, underpinning the polygenetic component of this disease. In the same study, they identified 13 new susceptibility loci for schizophrenia and replicated the *MHC* locus. Although the most replicated locus in schizophrenia is the *MHC* region, further investigations with regard to disease susceptibility have been difficult as this region is a rather complex composition of SNPs in high LD blocks across many genes involved in neurodevelopment, immunity, and other processes (Bergen and Petryshen 2012).

In the latest PCG GWAS (Schizophrenia Working Group of the Psychiatric Genomics C 2014), the sample size increased to nearly 37,000 cases and 113,000 controls, and 108 genome-wide significant loci were identified. Associated genes were enriched for genes expressed in brain regions, providing evidence for biological plausibility. It has to be noted that each of these 108 loci exhibits only a small effect, with risk-associated alleles differing mostly $<2\%$ in frequency between cases and controls.

Most of the schizophrenia-associated genes can be grouped into several categories: therapeutic targets (*DRD2* and *GRM3*), glutamatergic transmission (such as *GRIN2A* and *GRIA1*), neuronal calcium signaling (e.g., *CACNA1C* and *NRGN*), or synaptic function and plasticity (such as *MEF2C* and *CNTN4*). Interestingly, in independent studies, genes encoding calcium channels and genes involved in glutamatergic neurotransmission and synaptic plasticity have been shown to have implications in schizophrenia based on rare genetic variations (Purcell et al. 2014; Fromer et al. 2014; Kirov et al. 2012). Furthermore, GWAS hits identified in the PGC cohort significantly overlap with genes where de novo non-synonymous mutations in schizophrenia have been reported. In a combined meta-analysis of ~21,000 cases and ~20,000 controls, Marshall et al. (2017) found 8 genome-wide significant loci with copy number variants (CNVs). This suggests that both common and rare variations have implications in schizophrenia. In general, CNVs show generally greater effect sizes (odds ratios (ORs) of 4–70) on schizophrenia risk than common variants (OR < 1.3) (Birnbaum and Weinberger 2017).

Although common and rare risk loci have been identified, the causative pathophysiology of schizophrenia is still unknown. Gene expression and DNA methylation studies on postmortem brain suggest that risk factors seem to occur mainly

during early brain development and not at the stage of adolescent when the diagnosis is typically made (Birnbaum and Weinberger 2017).

3.2 Bipolar Disorder

Bipolar disorder (BD) presents with high heritability estimates between 80 and 90% (Craddock et al. 2005; McGuffin et al. 2003). Main symptoms of the disease are unpredictable mood swings between mania and depression (Kerner 2014). BD is divided into two subtypes: bipolar I disorder with manic episodes and bipolar II disorder with hypomanic episodes.

The first big GWAS on bipolar disorder was published in 2007 by the WTCCC (Wellcome Trust Case Control C 2007) with 1,900 BP cases and 3,000 controls. The authors identified one genome-wide significant hit in 16p1 which includes *PALB2* (partner and localizer of *BRCA2*), *NDUFAB1*, and *DCTN5*. Baum et al. (2008) used a DNA pooling approach followed by individual genotyping of interesting genetic markers; they identified one genome-wide significant hit in *DGKH*. Sklar et al. (2008) did not find genome-wide significant hits and however reported genome-wide significance in a haplotype test in *MYO5b* and furthermore associations with *CACNA1C*, consistent with WTCCC results. The study by Ferreira et al. (2008) also supported findings in *CACNA1C* as well as in *ANK3*. Scott et al. (2009) reported variants, albeit not genome-wide significant, in *MCTP1*, *GNL3*, *NEK4*, *ITIH*, and *ANK3*. Smith et al. (2009) ran GWAS independently in Europeans and African Americans. Although no genome-wide significant SNPs were reported, they found evidence for associations in *SLITRK2* and *NTRK2*. Several other GWAS were published (Cichon et al. 2011; Djurovic et al. 2010; Hattori et al. 2009; Lee et al. 2011b; Smith et al. 2011; Yosifova et al. 2011) until the PGC reported a GWAS on BD (Psychiatric GCBDWG 2011) comparing over 10,000 bipolar cases against over 50,000 controls in the combined initial and replication sample. They confirmed genome-wide significant evidence of association for *CACNA1C* and identified a new intronic variant in *ODZ4*. Chen et al. (2013) reported novel association findings near the genes *TRANK1*, *LMAN2L*, and *PTGFR*. Mühleisen et al. (2014) ran a GWAS on ~2,200 patients with BD and ~5,000 controls and identified 56 genome-wide significant hits including the previously reported risk loci *ANK3*, *ODZ4*, and *TRANK1*, as well as two new hits in *ADCY2* and a region between *MIR2113* and *POU3F2*. Hou et al. (2016) added *ERBB2* and an intergenic region on chromosome 9p21.3 and Charney et al. (2017) *ADD3* and *XPNPEP1* to the list of possible candidates. Ikeda et al. (2017a) reported novel genome-wide significant findings at 1q12.2, a region known to contain regulatory genes for plasma lipid levels (*FADS1/2/3*), in a Japanese population. A recent study by Stahl and colleagues (2019) identified 30 genome-wide significant loci including *CACNA1C*, *GRIN2A*, *SCN2A*, *SLC4A1*, *RIMS1*, and *ANK3*.

Three of the most striking findings of BD GWAS with regard to gene functionality are possibly *ANK3* and *CACNA1* which are associated with white-matter integrity and brain volume (Gurung and Prata 2015) and *FADS1/2/3* indicating that lipid abnormality may be associated with the pathophysiology of BD (Ikeda et al. 2017b). However, the exact disease etiology remains to be elucidated.

3.3 Major Depressive Disorder (MDD)

MDD is the most common psychiatric disorder with a lifetime risk of 10–20% and heritability estimates around 40% (Major Depressive Disorder Working Group of the Psychiatric GC et al. 2013). It is characterized by depressed mood, diminished interests, and impaired cognitive function (Flint and Kendler 2014).

Up to recently, it has been proven to be difficult to identify genome-wide significant susceptibility loci for MDD. The first GWAS (Major Depressive Disorder Working Group of the Psychiatric GC et al. 2013; Sullivan et al. 2009; Wray et al. 2012; Muglia et al. 2010; Shi et al. 2011b; Shyn et al. 2011; Lewis et al. 2010; Rietschel et al. 2010) did not report genome-wide significant findings. Kohli et al. (2011) found a genome-wide significant SNP close to *SLC6A15* when they combined their initial and replication sample and used a recessive model. The CONVERGE consortium found genome-wide significant hits in the *LHPP* gene and near the *SIRT1* gene (Consortium C 2015) in a Han Chinese population. However, these identified risk alleles are very rare (low frequency) in Europeans.

The first genome-wide significant hit in Europeans was reported in a meta-analysis study combining samples from the PGC consortium with large-scale consumer genetic data from 23 and Me (Hyde et al. 2016). In this study, 15 MDD-associated loci were reported including *LFM4*, *TMEM161B–MEF2C*, *MEIS2–TMCO5A*, and *NEGR1*. Going one step further, the PGC recently published a GWAS with a combined sample size of 130,664 MDD cases and 330,470 controls. Forty-four independent genome-wide significant loci were reported (Wray et al. 2018) including 30 novel hits. The most significant hits were located in or near *OLFM4* and *NEGR*; novel associations included *RBFOX1* and *LRFN5*. Genes that were targets of antidepressant medications were strongly enriched for MDD association hits indicating the relevance of these findings for pharmacotherapy.

MDD has a modest heritability pointing to the fact that both genetic and environmental factors are involved in disease etiology. Environmental factors, such as childhood abuse, are strongly associated with the risk of developing MDD (Flint and Kendler 2014). Furthermore, twin studies reported that environment accounts for over 60% of the variance (Sullivan et al. 2000). Thus, in future, probably, gene x environment (GxE) studies will become more and more important to elucidate disease pathology of MDD.

3.4 Attention Deficit Hyperactivity Disorder (ADHD)

Attention deficit hyperactivity disorder (ADHD) is a common childhood disorder affecting 2–10% of school-aged children worldwide (Hawi et al. 2015). Twin studies estimate a heritability of 70–80% (Franke et al. 2012; Faraone et al. 2005). ADHD is characterized by extreme levels of motor activity, impulsivity, and inattention. In 30–60% of the affected, these symptoms occur up into adulthood and are associated with increased risk for drug abuse, for instance (Mannuzza et al. 1993; Konstenius et al. 2015). Several GWAS been conducted for ADHD (Neale et al. 2008, 2010a, b; Hinney et al. 2011; Stergiakouli et al. 2012; Yang et al. 2013; Mick et al. 2010; Lesch et al. 2008; Middeldorp et al. 2016; Zayats et al. 2015; Sanchez-Mora et al. 2015; Alemany et al. 2015), but none reported any genome-wide significant hits. However, a trend for *CDH13* was observed in some of these studies. Recently, the PGC reported a genome-wide meta-analysis including over 20,000 ADHD cases and over 35,000 controls (Demontis et al. 2019). They identified 16 genome-wide significant loci including *FOXP2*, *DUSP6*, *SEMA6D*, and *MEF2C*.

3.5 Autism

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder characterized by various degree of abnormal communication, social reciprocity, and restricted repetitive behaviors (Lai et al. 2014). Twin studies estimate the heritability to be more than 80% (Ronald and Hoekstra 2011) with a prevalence of about 1% (Fernell and Gillberg 2010). It is believed that rare penetrant mutations as well as common variants with multiplicative effects contribute to a complex disorder like autism (Lai et al. 2014).

The earliest report of a pathogenic mutation involved the gene *NLGN4X* (Jamain et al. 2003; Laumonnier et al. 2004). Sebat et al. reported that large de novo structural variation was present in 10% of sporadic autism families (Sebat et al. 2007). However, common genetic variations explain about half of the genetic risk in ASD variants (Gaugler et al. 2014), and thus, GWAS are the ideal tool to elucidate associated variants. While some studies could not detect genome-wide significant loci (Ma et al. 2009; Salyakina et al. 2010; Kuo et al. 2015), the Autism Genome Project Consortium (AGP) reported one genome-wide significant hit in *MACROD2* (Anney et al. 2010), and Wang et al. (2009) found genome-wide significance in the region between *CDH9* and *CDH10*. Interestingly, Kerin et al. (2012) identified the noncoding RNA *MSNP1AS* in this region to be overexpressed in postmortem cerebral cortex of individuals with ASD. Xia et al. found genome-wide significant hits in *CSDE1*, *NRAS*, and *TRIM33* (Xia et al. 2014). Connolly et al. (2013) identified genome-wide significant loci in *KCND2*, *C8ORFK32*, *NOS2A*, *NELL1*, *BIN1*, *MPN2*, *SDK1*, and *FER*.

The PGC reported no genome-wide significant association (Autism Spectrum Disorders Working Group of The Psychiatric Genomics C 2017); top-associated loci included *CUEDC2*, *MACROD2*, and *ASTN2*. Importantly, they observed significant genetic correlation between ASD and schizophrenia pointing to a shared disease etiology.

3.6 Cross Disorders: ASD, ADHD, BP, MDD, and Schizophrenia

Genetic markers for psychiatric disorders do not uniquely map to only one diagnosis. Diseases are generally separated on the basis of their symptom patterns and course of illness, but pathogenic mechanisms of psychiatric disorders are largely unknown. Therefore, although diseases present with different symptoms, they could share, at least partially, the same genetic etiology.

Most of the cross-disorder studies investigated the relationship between two disorders. Several studies suggest overlap in the genetic liability of schizophrenia and BD (Smoller and Finn 2003; Maier et al. 1993), and genetic variants associated with both schizophrenia and BD have been identified (International Schizophrenia C et al. 2009; Schizophrenia Psychiatric Genome-Wide Association Study C 2011; Psychiatric GCBDWG 2011). Ruderfer et al. (2014) compared schizophrenic and bipolar patients and created a schizophrenia versus BD genetic risk score differentiating the two diseases. Furthermore, they ran a combined GWAS on schizophrenic/bipolar cases against healthy controls and found five genome-wide significant regions (*CACNA1C*, *IFI44L*, *MHC*, *TRANK1*, *MAD1L1*, and *PIK3C2A*). Also, genetic overlap between ASD and schizophrenia (King and Lord 2011; Levinson et al. 2011), especially in the context of CNVs, has been previously reported. Furthermore, also ASD and ADHD show a certain overlap (Ronald et al. 2008; Rommelse et al. 2010; Lichtenstein et al. 2010).

Huang et al. (2010) studied cross-disorder effects between MDD, BD, and schizophrenia and reported hits, although not genome-wide significant, close to *ADM* and in *NPAS3*.

In the first cross-disorder study focusing on five diseases, the PGC investigated shared genetic effects between ASD, ADHD, BD, MDD, and schizophrenia (Cross-Disorder Group of the Psychiatric Genomics C 2013). They ran a meta-analysis including in total over 33,000 cases and 27,000 controls and conducted a pairwise-cross-disorder-polygenic risk score analysis, defining risk scores for each disorder and applying these to the other disorders. The authors found significant overlap between MDD, schizophrenia, and BD, and also ASD, schizophrenia, and BD were significantly overlapping. Interestingly, no significant overlap between MDD and ASD or between ADHD and any other disorder was found. In the overall meta-analysis combining all five disorders, four independent regions with genome-wide significant hits were identified: *ITIH3*, *CACNB2*, *AS3MT*, and *CACNA1C*.

CACNA1C had already been identified as susceptibility gene for BD (Ferreira et al. 2008; Psychiatric GCBDWG 2011), schizophrenia (Schizophrenia Psychiatric Genome-Wide Association Study C 2011), and MDD (Green et al. 2010) and also in a cross-disorder study including BD and MDD (Liu et al. 2011). *CACNB2* was reported as susceptibility gene for BD in Han Chinese (Lee et al. 2011b), while *AS3MT* was previously associated with BD and schizophrenia (Schizophrenia Psychiatric Genome-Wide Association Study C 2011; Psychiatric GCBDWG 2011). Calcium channel signaling pathways were implicated across all five disorders, and SNPs associated across the disorders were also enriched for brain eQTL markers.

Along these lines, the PGC published another paper (Cross-Disorder Group of the Psychiatric Genomics C et al. 2013) which focused on the general genetic correlation between psychiatric disorders and further highlighted the genome-wide pleiotropy of these disorders. Pairwise genetic correlation between these disorders was estimated. This correlation is positive if cases of one disorder are more genetically similar to cases of another disorder than to their own controls. The genetic correlation using common SNPs was high between schizophrenia and BD; moderate between schizophrenia and MDD, BD and MDD, and ADHD and MDD; low between schizophrenia and ASD; and nonsignificant for other pairs of disorders as well as between psychiatric disorders and the negative control of Crohn's disease.

In future, disease treatment could be focused on a nosology informed by disease cause rather than by symptoms (Cross-Disorder Group of the Psychiatric Genomics C 2013). When looking into how different diseases correlate with each other, it is also interesting to learn more about their exact relationship (Docherty et al. 2016). Crespi and colleagues (Crespi et al. 2010) surveyed four possible models to reflect how schizophrenia and ASD might be related: ASD could be a subtype of schizophrenia ("subsumed model"); ASD could be a distinct disorder ("separate model"); both diseases could be diametric, i.e., opposite conditions ("diametric model"); or they could overlap to some distinct sharing some risk factors and phenotypes but not others ("overlapping model").

Newer approaches other than the classical GWAS have recently been applied to test for cross-disorder effects, e.g., phenotype-based approaches that look more closely into which genes are involved in multiple disorders (Gonzalez-Mantilla et al. 2016). In this context, a dimensional refinement of the phenotype (Docherty et al. 2016) and concepts like the Research Domain Criteria (RDoc) (Cuthbert and Insel 2013) will probably gain importance.

3.7 Posttraumatic Stress Disorder (PTSD)

Posttraumatic stress disorder (PTSD) is a debilitating psychiatric disorder that can occur after exposure to a potentially life-threatening traumatic event. To date only a handful of PTSD GWAS studies have been performed in humans. The first GWAS study in PTSD detected a genome-wide significant association between a SNP in the

retinoid-related orphan receptor alpha (*RORA*) gene and PTSD in a discovery sample (295 cases and 196 controls) of trauma exposed white non-Hispanic veterans and their intimate partners (Logue et al. 2013). The *RORA* gene plays an important role in protecting brain cells from injury, stress, and disease (Logue et al. 2013). The association of PTSD with the *RORA* gene has since then been replicated in a cohort of Florida hurricane survivors (Amstadter et al. 2013) but was not replicated in two independent replication samples (Guffanti et al. 2014).

Another GWAS was performed in an African American population (94 PTSD cases and 319 controls) of women who had been exposed to varying traumatic events (Guffanti et al. 2013). Only one SNP in a novel RNA gene *lincRNA AC068718.1* (rs10170218) reached genome-wide significance. This SNP association was replicated in a female European population (578 PTSD cases and 1,963 controls) and was found to be marginally significant. A third study performed a GWAS in 300 PTSD European Americans and 444 PTSD African Americans (Xie et al. 2013). The unknown SNP rs406001 reached genome-wide significance in the European American population, and no SNPs reached genome significance in the African American population. The genome-wide significant SNP was in a region with three other SNPs in LD, located about 630 kb downstream of the Cordon-Bleu (*COBL*) gene. The second significant locus from this study pointed toward the Tolloid-Like 1 or *TLL-1* in PTSD, a zinc-dependent metalloprotease that plays a key role in extracellular matrix remodeling (Apte and Parks 2015).

A further study employed a relatively large multiracial PTSD cohort of 2,312 Iraq and Afghanistan veterans (Ashley-Koch et al. 2015) and did not identify any individual SNPs that reached genome-wide significance. The study pointed toward several plausible candidate genes showing nominal significance including *TBC1D2*, *SDC2*, and *PCDH7*, genes previously implicated in neurologic processes.

A study using a small military cohort (Systems Biology PTSD Biomarkers Consortium, $N = 147$) identified a genome-wide significant SNP rs717947 at chromosome 4p15 associated with PTSD and replicated this finding in a larger urban community cohort (Grady Trauma Project, GTP, $N = 2{,}006$) (Almli et al. 2015). In the GTP replication sample, SNP rs717947 was associated with PTSD diagnosis in females ($N = 2{,}006$, $P = 0.005$) and was also found to be a methylation quantitative trait locus (meQTL) in the GTP replication sample ($N = 157$, $P = 0.002$). Further, the risk allele of rs717947 was also associated with decreased medial and dorsolateral cortical activation to fearful faces ($N = 53$, $P < 0.05$) in the replication sample. The peak of SNPs in LD with the genome-wide significant SNP on chromosome 4 was located in close proximity to the ncRNA *BC036345* whose function is unknown.

Another GWAS performed on samples from combat-exposed US Marines and Sailors from the Marine Resiliency Study (MRS) scheduled for deployment to Iraq and/or Afghanistan. This meta-analysis identified the phosphoribosyl transferase domain containing one gene (*PRTFDC1*) as a genome-wide significant PTSD locus (rs6482463), with a similar effect across ancestry groups (Nievergelt et al. 2015). Replication of the *PRTFDC1* signal with PTSD in an independent military cohort in the same study showed evidence for robustness of the finding.

One PTSD GWAS investigated two large US military cohorts with a total of around 10,000 samples (Stein et al. 2016). Genome-wide significance was reached for rs159572 in the *ANKRD55* gene, implicated in autoimmune and inflammatory disorders, but only in the African American samples.

One GWAS study examined symptoms defining the dissociative subtype of PTSD and found no SNPs reaching genome-wide significance but ten SNPs showing suggestive association (Wolf et al. 2014).

Given the modest GWAS results in the field of PTSD, great advances have been made via the formation of the Psychiatric Genomics Consortium-PTSD (PGC-PTSD) Workgroup in 2013 (Logue et al. 2015). To facilitate the analysis of different types of data, focus groups have been created within the PGC-PTSD Workgroup including the PGC-PTSD Epigenetics Workgroup and the PGC-PTSD Neuroimaging Genetics Workgroup among others (Logue et al. 2015). The most recent largest PTSD GWAS from the PGC-PTSD included data from 20,730 individuals across 11 multiethnic studies (Duncan et al. 2017). In addition to identifying risk loci for PTSD, the authors quantified PTSD heritability and examined shared genetic risk of PTSD with schizophrenia, BD, and MDD to identify risk loci for PTSD. The study did not reveal any significant genome-wide hits in European Americans and 13 genome-wide significant hits in African Americans (Duncan et al. 2017). SNP-based heritability estimates for European American females were 29%, similar to that for schizophrenia, and were substantially higher than that in European American males (not different from zero). Using polygenic risk score profiling, there was strong evidence of overlapping genetic risk between PTSD and schizophrenia along with more modest evidence of overlap of PTSD with BD and MDD.

In summary, a handful of scattered GWAS in PTSD have been conducted, and several candidate genes have been revealed. Further larger GWAS analyses within the PGC-PTSD consortium are currently underway and are likely to identify additional candidates for PTSD.

3.8 Obsessive-Compulsive Disorder (OCD)

Obsessive-compulsive disorder (OCD) is characterized by recurring obsessions and/or compulsions, affects up to 2% of the world's population, and is 4.5 times more common in males than females (Eaton et al. 2008). The success of GWAS in OCD has been limited, with no genome-wide significant SNPs identified so far; some of the largest OCD GWAS studies are reviewed below.

The GWAS from the International OCD Foundation Genetics Collaborative (IOCDF-GC) was performed in 1,465 cases, 5,557 ancestry-matched controls, and 400 trios (Stewart et al. 2013). The trio analysis identified the SNP rs6131295, near *BTBD3*, that exceeded the genome-wide significance threshold, but this SNP lost genome-wide significance when meta-analyzed with the case-control samples. For the top-ranked SNPs ($P < 0.01$) from the trio analysis, a significant enrichment of

methylation QTLs and frontal lobe expression quantitative trait loci (eQTLs) was observed, suggesting a role in brain gene expression and OCD etiology.

The OCD Collaborative Genetics Association Study (OCGAS) GWAS was conducted in a total of 1,065 families (including 1,406 OCD patients), combined with population-based samples, comprising a total sample of 5,061 individuals (Mattheisen et al. 2015). Although this study failed to detect any genome-wide significant hits, a trend for significance was seen for a SNP near *PTPRD*, a presynaptic gene that promotes the differentiation of glutamatergic synapses.

The recent largest GWAS was a meta-analysis of the two previous IOCDF-GC and OCGAS GWAS, investigating a total of 2,688 Europeans and 7,037 genetically matched controls (International Obsessive Compulsive Disorder Foundation Genetics C, Studies OCDCGA 2017). None of the SNPs reached genome-wide significance. SNPs located within or near the genes *ASB13*, *RSPO4*, *DLGAP1*, *PTPRD*, *GRIK2*, *FAIM2*, and *CDH20*, identified in linkage peaks and the original GWASs, were among the top signals. The common SNP heritability in the combined sample was estimated to be 0.28.

3.9 Alcohol, Nicotine, and Cannabis Dependence

A large number of GWAS have been performed on alcohol and other substance dependence traits; details of these studies are available on the GWAS catalog website (MacArthur et al. 2017). Some of the largest studies are described in this section.

Alcohol Dependence

With a lifetime prevalence of 12.5% in the general American population, alcohol dependence (AD) is a common, complex psychiatric disorder characterized by excessive and compulsive use of alcohol, which often results in physical and social consequences (Hasin et al. 2007). Significant advances in the identification of risk loci for AD can be largely attributed to the advent of genome-wide association studies (GWAS), and these studies have been reviewed in-depth (Hart and Kranzler 2015; Stickel et al. 2017). The first GWAS of AD was performed in a sample of German men, comprising a discovery sample of 476 cases and 1,358 controls (Treutlein et al. 2009) and a replication sample of 1,024 AD cases and 996 controls. Although no SNPs were genome-wide significant in the discovery sample, two SNPs that mapped to the *PECR* gene reached genome-wide significance in a meta-analysis of the discovery and replication samples. A total of 15 SNPs located in or near several genes (*CAST*, *ERAP1*, *PPP2R2B*, *ESR1*, *CCD41*, *ADH1C*, *GATA4*, and *CDH13*) were genome-wide significant in the meta-analysis across the two samples.

Another GWAS (Frank et al. 2012) in a German sample of 1,333 men with severe AD and 2,168 controls identified one genome-wide significant SNP (rs1789891) located between *ADH1B* and *ADH1C*, two alcohol-metabolizing enzyme genes. Furthermore, this was the first AD GWAS to identify a robust genome-wide

significant hit that was replicated in an independent sample and the first study to identify significant associations in alcohol-metabolizing enzyme genes, thereby replicating the findings from previous candidate gene studies (Bierut et al. 2012; Edenberg 2007; Olfson and Bierut 2012; Thomasson et al. 1991).

A GWAS performed by Gelernter et al. (2014) was the first study to impute the genotyped SNPs tested to include over nine million in African Americans and over six million in European Americans. The authors tested for association with a quantitative AD phenotype while controlling for cocaine, opioid, and nicotine dependence criteria in the sample and meta-analyzed the results with those from the SAGE sample (Bierut et al. 2010) and also with their discovery and replication samples. In African Americans, they identified several genome-wide hits at the alcohol dehydrogenase (*ADH*) locus on chromosome 4, with the strongest finding in *ADH1B* (rs2066702). In European Americans, the strongest finding was also in *ADH1B* (rs1229984). Both *ADH1B* SNPs were missense polymorphisms, and both associations replicated in an independent sample. Thus, as in other AD GWAS (Frank et al. 2012; Park et al. 2013), the ADH gene cluster had the greatest effect on AD risk.

To summarize, several genes including those within the ADH locus have been identified for AD.

Nicotine Dependence

Nicotine dependence is highly comorbid with several psychiatric disorders. The Tobacco and Genetics Consortium meta-analyses of several smoking phenotypes ($n = 74{,}053$) identified three loci associated with number of cigarettes smoked per day (Tobacco, Genetics C 2010). The strongest association was a SNP in the nicotinic receptor gene *CHRNA3* (rs1051730). Two other SNPs on chromosome 10 (rs1329650 and rs1028936) and one SNP in *EGLN2* (rs3733829) also exceeded genome-wide significance for cigarettes smoked per day. For smoking initiation, eight SNPs exceeded genome-wide significance, with the strongest association at a non-synonymous SNP in *BDNF* on chromosome 11 (rs6265). One SNP located near *DBH* on chromosome 9 (rs3025343) was significantly associated with smoking cessation.

In another GWAS (Gelernter et al. 2015), the chromosome 7 intergenic SNP rs13225753 was associated with nicotine dependence in Europeans Americans. In African Americans, associations were observed at several SNPs mapping to a region on chromosome 14 and two regions on chromosome 8. This study did not identify any associations at the chromosome 15 nicotinic receptor gene cluster (*CHRNA5-CHRNA3-CHRNB4*) previously associated with nicotine dependence and smoking quantity traits.

In a recently conducted largest-ever GWAS meta-analysis, the *CHRNA5-CHRNA3-CHRNB4* genes were reconfirmed to be associated with nicotine dependence, and a further novel association in the DNA methyltransferase gene *DNMT3B* was identified (Hancock et al. 2017).

Cannabis Dependence

After nicotine, cannabis is the most widely abused drug worldwide, and cannabis use is also a risk factor for psychiatric disorders including MDD (Lynskey et al. 2004) and schizophrenia (Power et al. 2014). To date, only three genome-wide studies have been conducted for cannabis-related phenotypes. The first GWAS (Sherva et al. 2016) uncovered three independent regions with genome-wide significant SNPs including rs143244591 in novel antisense transcript *RP11-206M11.7*, rs146091982 in the solute carrier family 35 member G1 gene (*SLC35G1*), and rs77378271 in the CUB and Sushi multiple domains 1 gene (*CSMD1*).

The International Cannabis Consortium meta-analysis of genome-wide association data of 13 cohorts ($N = 32{,}330$) and 4 replication samples ($N = 5{,}627$) did not find any individual SNPs reaching genome-wide significance, but gene-based tests identified four genes (*NCAM1*, *CADM2*, *SCOC*, and *KCNT2*) significantly associated with lifetime cannabis use (Stringer et al. 2016). This study demonstrated that the common SNPs explained 13–20% of the liability of lifetime cannabis use and indicated a strong genetic correlation between lifetime cannabis use and lifetime cigarette smoking ($rg = 0.83$).

Another recent meta-analysis of genome-wide association study data on 2,080 cannabis-dependent cases and 6,435 cannabis-exposed controls of European descent which identified a cluster of correlated SNPs in a novel region on chromosome 10 was genome-wide significant (Agrawal et al. 2017). The SNPs were located nearby multiple genes that are expressed in brain-derived tissues. Among the SNPs, rs1409568 was predicted to modify binding scores for several transcription factors, had active enhancer marks for addiction-relevant brain regions including the dorsolateral prefrontal cortex and the angular and cingulate gyri, and was also associated with a modest increase in right hippocampal volume (2.13%) in an independent sample.

3.10 Personality Traits: Neuroticism and Borderline Personality

Personality traits are influenced by genetic and environmental factors (Vukasovic and Bratko 2015) and have been reported to be associated with mental health (Lo et al. 2017). Genetic research of personality traits is still in its infant stages compared to other psychiatric disorders with early genetic studies performed in small samples and focused on single candidate genes. This section outlines GWAS studies in neuroticism and borderline personality traits in conjunction with other psychiatric disorders or symptoms.

3.10.1 GWAS of Neuroticism

A meta-analysis of the Genetics of Personality Consortium identified a novel locus (rs35855737) for neuroticism in *MAGI1*, a known gene that has been previously been associated with bipolar disorder and schizophrenia (Genetics of Personality C et al. 2015). Additionally, the researchers found that common genetic variants explained 15% of the variance in neuroticism and the polygenic risk score for neuroticism significantly predicted MDD in independent cohorts, providing evidence for a genetic overlap.

Another GWAS of personality traits discovered 3 genetic variants associated with subjective well-being, 2 variants associated with depressive symptoms, and 11 variants associated with neuroticism (Okbay et al. 2016). This study also found a genetic overlap across subjective well-being, depressive symptoms, and neuroticism (rg = −0.81 between subjective well-being and depressive symptoms, rg = −0.75 between subjective well-being and neuroticism, and rg = 0.75 between depressive symptoms and neuroticism).

Lo and colleagues conducted a meta-analysis of GWAS and identified six genetic loci, (five novel) significantly associated with personality traits (Lo et al. 2017). Among the traits, extraversion was associated with genetic variants in *WSCD2* and near *PCDH15*, and neuroticism was associated with genetic variants in *L3MBTL2* and on chromosome 8p23.1. The study also found high genetic correlations between neuroticism and MDD (rg = 0.54), between extraversion and attention deficit hyperactivity disorder (rg = 0.30), and between openness and schizophrenia (rg = 0.36) and BD (rg = 0.34) among others.

A recent study investigated over 329,000 individuals from the UK Biobank and reported 116 significant independent loci associated with neuroticism, 15 of which were also replicated in an unrelated cohort of over 122,000 individuals (Luciano et al. 2018). The study revealed significant genetic correlations between neuroticism and depressive symptoms (rg = 0.82), MDD (rg = 0.69), and subjective well-being (rg = −0.68,) together with other mental health traits.

3.10.2 GWAS of Borderline Personality

To the best of our knowledge, only two GWAS in borderline personality have been reported so far, one based on borderline personality features (Lubke et al. 2014) and the other based on borderline personality disorder (Witt et al. 2017). Although no genome-wide significant loci were identified, the first GWAS found seven SNPs within a promising locus corresponding to myelination-protein *SERINC5* (chromosome 5) to be associated with borderline personality features; these results were also confirmed in an independent sample (Lubke et al. 2014). The borderline personality disorder GWAS was performed in 998 patients and 1,545 controls (Witt et al. 2017). While no SNP survived multiple-testing correction for association, gene-based analysis yielded two significant genes *DPYD* and *PKP4*, both previously implicated

in bipolar disorder and schizophrenia. *SERINC5*, which was the top hit of the previous GWAS of borderline personality features (Lubke et al. 2014), showed nominal significance in the study. This was the first study to demonstrate the genetic overlap of borderline personality disorder with BD (rg = 0.28), schizophrenia (rg = 0.34), and MDD (rg = 0.57).

In summary, both studies pointed toward the role of *SERINC5* among others, in borderline personality traits. The *SERINC5* protein is enriched in myelin in the brain and incorporates serine into newly forming membrane lipids (Krueger et al. 1997). This is of interest, given the previous findings that decreased myelination is associated with a reduced capacity for social interaction (Lubke et al. 2014; Liu et al. 2012).

4 Genome-Wide Association Studies in Psychiatric Disorders: Future Directions

GWAS allow the interrogation of common genetic polymorphisms in the genome without making any assumptions about the genomic location of the causal variants. Despite the large number of loci that have been identified, for most diseases, only a modest fraction of the predicted heritability has been accounted for through GWAS. Given the effects of natural selection and that genetic variants having a major effect on overall disease susceptibility would decrease reproductive fitness and would hence be maintained at a low frequency within populations has raised questions about the ability of GWAS methods to identify major risk loci for complex diseases.

The potential and limitations of GWAS have recently been reviewed in-depth (Visscher et al. 2017). GWAS methods have been limited by a variety of factors; hypothesis-free scanning of the genome, for instance, has the potential to identify many false associations, and therefore replication and/or validation in independent populations is essential. There is also no strict consensus on the statistical correction for multiple tests, and different methods and strategies including a two-step study design have been suggested (Wason and Dudbridge 2012). An additional challenge for GWAS is that it is currently based on the "common disease, common variant" (CDCV) hypothesis, which means that rare susceptibility variants are generally not detected. GWAS findings also typically point toward a region of linkage disequilibrium spanning several hundreds or thousands of base pairs harboring sets of correlated SNPs; hence, the risk SNPs implicated by GWAS are not necessarily causal but instead are more likely in linkage disequilibrium (LD) with true causal variants, and/or these intergenic and intronic risk variants represent regulatory elements. Thus, it is essential to demonstrate biological, functional, and clinical relevance for these GWAS loci via studies that confirm a functional link between a SNP and a variant in LD with the GWAS SNP with the disease (Edwards et al. 2013). Nevertheless, owing to the lack of tools and our limited knowledge, it has not been straightforward to shift from the GWAS loci to the underlying biological

mechanisms nor has it been easy to identify the gene(s) involved in the susceptibility to the disease. Finally, given the complex nature of psychiatric disorders with both common and rare genetic variants as well as environmental factors contributing to risk, complex and sophisticated statistical algorithms such as those described in this chapter are required to predict susceptibility to disease.

Despite the many challenges of GWAS, these studies have uncovered valuable biological insights about genes and pathways involved in psychiatric disorders. Newer approaches in the future will advance our understanding of disease pathophysiology with a combination of deeper resequencing of loci, imputation, assessment of copy number variations, insertions/deletions, epigenetics, and gene expression in larger, well-characterized samples, to account for the missing heritability in psychiatric disorders. The growth of large population biobanks with extensive genetic and phenotype information such as the 1000 Genomes project (Genomes Project et al. 2015) and the UK biobank (Ollier et al. 2005) will also aid to identify less common genetic variants associated with psychiatric disorders and behavioral traits; such efforts are currently underway and reflect the next wave of genetic discovery. Other projects such as the ENCODE (Consortium 2012) and Roadmap Epigenomics projects (Roadmap Epigenomics et al. 2015) have already greatly facilitated our understanding of gene regulation and have provided the tools for studying gene regulation and function, to unravel the biology underlying GWAS associations. Other complementary approaches to GWAS such as phenome-wide association studies (PheWAS) incorporate methods that conduct a genotype-to-phenotype analysis have been useful in immune-related disorders (Denny et al. 2010), and such methods might add value when applied to psychiatric disorders.

GWAS findings can also have impact on personalized medicine (Smoller 2014) by identifying genetic variants associated with the disease, and hence pointing to new drug targets, by stratifying patients into different therapeutic subgroups and pharmacogenetics studies investigating if genetic variants are associated with response. While the discovery of drugs in psychiatric disorders via GWAS is still in its infant stage, there is potential for the role of genetics and novel genes identified to impact on drug discovery.

In summary, GWAS in psychiatric disorders have shed light on a number of novel genes that might play a role in the pathophysiology of the disorders. Given the heterogeneous nature of psychiatric disorders, it may be more important to identify and clearly define patients within each biological or clinical subtype before utilizing resources toward more extensive and expensive genetic studies. Systems approaches that combine different layers of genomics information and provide a greater understanding of gene function and biological etiology of psychiatric disorders will shed light on the molecular underpinnings of psychiatric disorders.

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Genetic Variation in Long-Range Enhancers

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Abstract *Cis*-regulatory elements (CREs), including insulators, promoters, and enhancers, play critical roles in the establishment and maintenance of normal cellular function. Within each cell, the 3D structure of chromatin is arranged in specific patterns to expose the CREs required for optimal spatiotemporal regulation of gene expression. CREs can act over large distances along the linear genome, facilitated by looping of the intervening chromatin to allow direct interaction between distal regulatory elements and their target genes. A number of pathologies are associated with dysregulation of CRE function, including developmental disorders, cancers, and neuropsychiatric disease. A majority of known neuropsychiatric disease risk loci are noncoding, and increasing evidence suggests that they contribute to disease through disruption of CREs. As such, rather than directly altering the amino acid content of proteins, these variants are instead thought to affect where, when, and to what extent a given gene is expressed. The distances over which CREs can operate often render their target genes difficult to identify. Furthermore, as many risk loci contain multiple variants in high linkage disequilibrium, identification of the causative single nucleotide polymorphism(s) therein is not straightforward. Thus, deciphering the genetic etiology of complex neuropsychiatric disorders presents a significant challenge.

Keywords Disease · Enhancers · Epigenome · Transcription

1 Introduction

The human body is made up of hundreds of different cell types, each of which shares an essentially identical genome. Human nuclei are typically between 2 and 10 μm in diameter and contain approximately 2 m of DNA. In order to fit inside the nucleus, DNA is packaged into chromatin. This packaging is not random and differs between cell types, leading to the exposure of the distinct repertoire of *cis*-regulatory elements (CREs) required to generate cell-type-specific transcriptomes (reviewed in Cremer and Cremer 2001; Bulger and Groudine 2010; Heinz et al. 2015; Romanoski et al. 2015). Thus, the spatial organization of the genome plays a key role in the regulation of gene expression, the disruption of which may lead to disease (Dekker and Mirny 2016; Dixon et al. 2016; Lupianez et al. 2016).

2 Structure of Chromatin and 3D Genome

The fundamental unit of chromatin organization is the nucleosome, an octamer of histone proteins, which accommodates approximately 150 nucleotides of DNA that wrap around the complex. Nucleosomes coalesce into chromatin fibers, which are further compacted into chromosomes. Chromosomes are highly condensed during mitosis but become de-condensed during the interphase of the cell cycle. The

conformation of the genome during interphase, which comprises the major fraction of the cell cycle, during which a cell grows and expresses its genes, is not disorganized but highly structured. The three-dimensional (3D) structure of the genome is implicated in cell-type-specific regulation of gene transcription by facilitating contact between distal regulatory elements called enhancers and target promoters. In 1885, Carl Rabl proposed the "chromosome territory" model, which held that each chromosome occupies a defined volume of the nucleus and only shares nuclear space with a small number of adjacent chromosomes (Rabl 1885). This model was not validated until the 1980s, when Cremer and co-workers induced local DNA damage in the nucleus using a focused laser and, by providing radioactively labeled nucleotides to mark the chromosomal regions as the cells repaired the DNA damage, were able to observe DNA damage on only a few chromosomes (Cremer et al. 1982). Subsequent studies using fluorescent in situ hybridization confirmed that chromosomes occupy distinct regions within the nucleus during interphase (Cremer and Cremer 2001; Habermann et al. 2001; Parada et al. 2002, 2004; Tanabe et al. 2002; Meaburn and Misteli 2007).

The genome is broadly partitioned into megabase-sized compartments that consist of gene-rich regions of open chromatin, corresponding to transcriptionally active regions and, conversely, gene-poor regions of closed chromatin, corresponding to transcriptionally silent regions (Lieberman-Aiden et al. 2009). The genome is further segregated into locally self-interacting neighborhoods that are insulated from each other by boundary elements enriched for the architectural protein CTCF (Dixon et al. 2012; Rao et al. 2014). These neighborhoods, referred to as topologically associated domains (TADs), are cell-type invariant and conserved from mouse to human. The boundary elements insulating TADs are enriched for housekeeping genes, suggesting that other factors, in addition to CTCF, may serve as barriers in genomic organization. TAD boundaries are also known to play a role in gene expression, as deletion or disruption of boundary elements promotes aberrant enhancer-promoter interactions across adjacent TADs, leading to defective transcription (Nora et al. 2012; Lupianez et al. 2015). Indeed, disruption of TAD boundaries can produce severe physiological defects. Genomic deletions and inversions causing human limb malformation were shown to disrupt TAD boundary elements, an observation that was replicated in CRISPR-/Cas9-modified mice (Lupianez et al. 2015). Thus, TADs are evolutionarily conserved and regulate enhancer-promoter specificity.

3 Structural Organization of CREs

Within TADs, chromatin structure is variable depending on cell type and cell context. The functional diversity of cells is accomplished, in part, through regulatory elements residing in regions of open chromatin that determine isoform selection of genes during transcription and the timing and extent of their expression (Heinz et al. 2015). In 1981, Banerji and colleagues described a DNA element capable of enhancing transcription in HeLa cells (Banerji et al. 1981). This activity was

mediated by a (virally derived) 72 bp repeat sequence that could act in either orientation and in multiple positions relative to the transcription start site (TSS). The same group subsequently described the first mammalian enhancer (Banerji et al. 1983). This element was shown to influence expression of the beta-globin gene and could function over a broad range of genomic distances (several hundred to several thousand bp, either up- or downstream of the promoter). The element also displayed cell-type specificity as, among the cell lines tested, it was only functional in lymphocyte derived cells. A subsequent boom in enhancer identification occurred, and their mechanism of action in numerous biological processes began to emerge (Shlyueva et al. 2014).

Although the human genome contains hundreds of thousands of potential regulatory elements, only a subset is active in a given cell type at any time (ENCODE Consortium 2012). The distribution of CREs throughout the genome, therefore, provides an additional layer of transcriptional regulation in eukaryotic cells, as loop structures within chromatin facilitate direct and specific interactions between otherwise distal DNA elements, often separated by many kilobases along the linear genome (Gaszner and Felsenfeld 2006; Sanyal et al. 2011, 2012; Benabdallah et al. 2016). The 3D genome differs between cell types, is dynamic, and can change both during development and in response to external stimuli. Regions of open chromatin are enriched in enhancers, which facilitate the specificity of gene expression through interactions with sequence-specific transcription factors (TFs) (Vernimmen and Bickmore 2015). Each gene may be regulated by multiple enhancers depending on the cellular context. Similarly, a single enhancer can also contribute to the expression of a number of different genes, in some cases even if those genes reside on different chromosomes (Spilianakis et al. 2005). Thus, the manner and extent to which the genome is utilized is pivotal in defining the specifics of cellular identity and function.

4 Implication of CREs in Disease

Numerous studies link dysregulation of the epigenome to disease (e.g., see Robertson 2005; Williamson et al. 2011; Ward and Kellis 2012; Spielmann and Klopocki 2013; Spielmann and Mundlos 2013; Shen et al. 2014; Albert and Kruglyak 2015; Mirabella et al. 2015; Chatterjee and Ahituv 2017) and genetic variation within the noncoding, regulatory regions of the genome have been associated with a growing list of diseases (Epstein 2009; Manolio et al. 2009; Parker et al. 2013; Scacheri and Scacheri 2015), including developmental disorders (Benko et al. 2009; Gordon et al. 2009), heart disease (Goring et al. 2007; Postma et al. 2016), cancer (Herz et al. 2014; Northcott et al. 2014; He et al. 2015; Khurana et al. 2016; Lin et al. 2016), diabetes (Scott et al. 2007), Crohn's (Libioulle et al. 2007), and asthma (Moffatt et al. 2007; Manolio et al. 2008). In addition, defects in higher-order chromatin structure have also been implicated in disease. For example, mutations

within the cohesin complex (Deardorff et al. 2012; Gervasini et al. 2013), which functions to facilitate interactions between distal promoters and enhancers (Kagey et al. 2010), have been associated with Cornelia de Lange syndrome, which is characterized by physical and cognitive defects.

In the context of neuropsychiatric disease risk, both genetic and environmental factors are known to contribute (Gandal et al. 2016). Genome-wide association studies (GWAS) of complex neuropsychiatric diseases, such as schizophrenia (SCZ; Schizophrenia Working Group of the Psychiatric Genomics 2014), bipolar disorder (BD) (Stahl et al. 2018), Alzheimer's disease (AD) (Lambert et al. 2013), and major depressive disorder (Wray et al. 2018), have identified numerous risk loci, most of which are noncoding. Examining over 35,000 cases and 110,000 controls, the Schizophrenia Working Group of the Psychiatric Genomics Consortium identified 108 genome-wide significant loci associated with increased risk of SCZ (Schizophrenia Working Group of the Psychiatric Genomics 2014). This list has subsequently been expanded, to include over 200 risk loci (ongoing analysis by PGC3), and is expected to grow further with additional studies. The majority of the identified loci are noncoding and are enriched in functional elements including enhancer sequences detected in human brain tissue (Gusev et al. 2014; Roussos et al. 2014). SCZ risk loci also co-localize with expression quantitative trait loci (eQTLs) (in particular, brain-derived eQTLs) (Roussos et al. 2014; Fromer et al. 2016; Hauberg et al. 2017), thereby implicating specific genes. However, these efforts have limited spatiotemporal resolution as most studies performed, thus far, have been restricted to homogenate brain tissue or include only broadly defined neuronal and non-neuronal populations (Fullard et al. 2017). As it is known that CREs display tissue- and cell-type specificity, with variants therein often only affecting those cells and tissues relevant to a given disease (Maurano et al. 2012; Trynka et al. 2013), an imperative of future studies will be to employ cell-type-specific or single-cell assays in order to more comprehensively understand the impact of these variants. For instance, SCZ-associated genetic loci are enriched within promoter and enhancer regions of neuronal cells (Roussos et al. 2014; Fullard et al. 2017). Furthermore, variants located in H3K4me3 (a marker of active promoters) sites specific to neuronal cells were more abundant when compared to those of non-neurons (Tansey and Hill 2018). In the case of AD, single nucleotide polymorphisms (SNPs) associated with increased risk for disease (Lambert et al. 2013) have been shown to be enriched at sites of open chromatin in immune cells and microglia (Tansey et al. 2018). These open chromatin sites contain DNA-binding motifs for specific TFs, including SPI1 and MEF2. Taken together, these observations are consistent with the fact that regulatory elements display tissue- and cell-type specificity. As such, identifying the specific cell types affected by a given variant will be an important step toward a more comprehensive understanding of the genetic etiology of neuropsychiatric disease.

5 Large-Scale Efforts to Study the Noncoding Genome

A number of large-scale efforts have sought to examine the role played by the noncoding genome in the regulation of cellular function. The ENCyclopedia Of DNA Elements (ENCODE) project (ENCODE Consortium 2012), the NIH Roadmap Epigenome Mapping Consortium (REMC) (Bernstein et al. 2010; Roadmap Epigenomics et al. 2015), and FANTOM5 (FANTOM Consortium 2014) have made great strides toward systematically cataloguing CREs within the human genome. Unfortunately, much of this data is not readily applicable to the study of gene expression in neuropsychiatric disease. The ENCODE project did not include brain tissue but focused, instead, on a variety of actively dividing cell lines and tissues. Although both REMC and FANTOM5 did include brain specimens, their use was limited to homogenate tissue isolated from controls. Within brain tissue, however, the different subclasses of neurons coexist alongside other cell types, including microglia, oligodendrocytes, and astrocytes. Since CRE-mediated transcriptional regulation has been shown to be cell-type specific (Heintzman et al. 2009; Cheung et al. 2010; Maurano et al. 2012; Roadmap Epigenomics et al. 2015), data derived from such studies is of limited use when applied to the study of a tissue as complex as the human brain. SCZ-associated abnormalities have been demonstrated in specific populations of brain cells, including neocortical neurons (Benes and Berretta 2001), astrocytes (Schnieder and Dwork 2011; McCullumsmith et al. 2015), oligodendrocytes (Haroutunian et al. 2014; Roussos and Haroutunian 2014; Mighdoll et al. 2015), and microglia (Bernstein et al. 2015). As such, the study of homogenate tissue samples may fail to distinguish signals unique to specific cell types, potentially missing critical changes in less abundant cell populations. Large-scale collaborative projects such as PsychENCODE (Akbarian et al. 2015) will attempt to address this issue by applying state-of-the-art omics approaches to specific cell populations (neurons and non-neurons in the case of PsychENCODE), in an effort to further our understanding of the complex genetic mechanisms that contribute both to normal brain function and to disease (see below).

6 Approaches to Detect Enhancer Sequences

Although the full extent to which the 3D structure of chromatin influences cell function remains unclear (Cattoni et al. 2015), the advent of methodologies that incorporate chromatin immunoprecipitation and next-generation sequencing technologies (ChIP-seq) is likely to further our understanding of this critical regulatory process (Fullwood et al. 2009; Fullwood and Ruan 2009). ChIP-seq utilizes antibodies against epigenetic markers and can be used to assess enrichment for histone 3Lys4 tri-methylation (H3K4me3), histone 3Lys27 acetylation (H3K27ac), and histone H3 at lysine 4 mono-methylation (H3K4me1) to identify putative active promoters and enhancers and active or primed enhancers, respectively.

Cap analysis of gene expression (CAGE) facilitates fine mapping of TSSs and promoter regions by sequencing the 5′ end of mature RNA (Shiraki et al. 2003; Takahashi et al. 2012). In CAGE-seq, RNA is reverse-transcribed and the 3′ ends biotinylated. Nonhybridized, single-stranded RNAs are removed by digestion with RNase, leaving 5′ complete cDNAs that are captured using streptavidin and subjected to next-generation sequencing. The FANTOM5 project has applied CAGE-seq to a wide array of cells and tissues from human and mouse, including the brain. In so doing, they have identified and quantified the activity of at least one promoter for more than 95% of annotated protein-coding genes in the human reference genome (FANTOM Consortium 2014). In addition, using H3K27ac and H3K4me1 ChIP-seq data from the ENCODE project (ENCODE Consortium 2012), FANTOM5 also applied CAGEseq data to identify a range of enhancers across human cells, through the detection of enhancer RNAs (eRNA) (Andersson et al. 2014). eRNAs are transcribed in proportion to enhancer activity, and their levels correlate with those of mRNA from nearby genes and have been shown to be differentially expressed in SCZ (Hauberg et al. 2018).

The nucleosome is known to play a central role in mediating gene expression and exists in a dynamic equilibrium between open and closed states (Mellor 2005). Nucleosome rearrangement at promoters and enhancers leads to open chromatin states and results from the binding of specific regulatory factors (Henikoff 2008). Open or accessible regions of the genome are regarded as primary positions for regulatory elements and, as such, play a critical role in regulating transcription (John et al. 2011). Approaches such as DNase-seq (DNase I 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 have largely been superseded by methods that require lower amounts of input material, an important consideration when working with precious biological samples of limited availability. More recently, a tagmentation-based method called the Assay for Transposase-Accessible Chromatin followed by Sequencing (ATAC-seq) has been developed (Buenrostro et al. 2013). ATAC-seq employs a transposome complex to insert oligonucleotides into accessible regions of the genome. This facilitates the generation of sequencing libraries enriched for open chromatin with sufficient resolution to map TF occupancy and nucleosome positions in regulatory sites (Buenrostro et al. 2015a). Subsequent iterations of the method allow for chromatin structure to be profiled in as few as 500 cells (Corces et al. 2017) and those isolated from 50 μm sections and frozen archival material (including human brain (Egervari et al. 2017; Fullard et al. 2017)). In addition, the approach has been further optimized to allow chromatin profiling at the single-cell level (Buenrostro et al. 2015b; Lake et al. 2018).

7 Approaches to Detect the Long-Range Enhancers

Several methods based on chromosome conformation capture (3C) have been developed to assess the frequency at which any two loci in the genome are in close enough physical proximity to functionally interact (Dekker et al. 2002; Zhao et al. 2006; Dostie et al. 2006). 3C involves cross-linking of interacting DNA segments, followed by digestion with a frequently cutting restriction enzyme. Digested DNA is then religated, 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. 3C can detect interactions between distal genetic elements, e.g., between a gene and an enhancer (Simonis et al. 2007; Naumova et al. 2012), and can range from target-specific (3C) to unbiased, genome-wide approaches (Hi-C) (Dekker et al. 2013), including those at the resolution of single cells (Flyamer et al. 2017; Ulianov et al. 2017). Importantly, a number of these approaches have been successfully applied to studies of the human brain (Mitchell et al. 2014; Roussos et al. 2014; Won et al. 2016).

Alterations in chromosomal loop structures have been implicated in neuropsychiatric disease (Bharadwaj et al. 2013, 2014; Roussos et al. 2014). Bharadwaj and colleagues mapped the 3D configuration of a 200 kb stretch of the human genome containing the GAD1 GABA synthesis enzyme gene locus (Bharadwaj et al. 2013), which has previously been implicated in neuropsychiatric disease (Addington et al. 2005; Straub et al. 2007). This led to the identification a 50 Kb loop structure between noncoding intergenic DNA elements and the TSS of the gene encoding GAD1. This loop was further identified in an induced pluripotent stem cell-derived neuronal cell line when compared to skin fibroblasts or undifferentiated pluripotent stem cells, indicating the cell-type specificity of the structure. The loop was decreased in the prefrontal cortex of subjects with SCZ with a concurrent decrease GAD1 expression when compared with controls.

Variations in DNA sequence can have a profound effect on the function of distal regulatory elements. Bharadwaj and co-workers have also described a conserved, methyltransferase dependent loop structure in the prefrontal cortex involved in regulating the expression of the NMDA glutamate receptor, GRIN2B (Bharadwaj et al. 2014). Disruption of the loop resulted in decreased GRIN2B expression and led to impaired cognitive function and working memory defects in a mouse model. In the above examples, the loop structure is conserved between human and mouse, indicating their functional importance.

Disruption of long-range enhancer function has also been implicated in SCZ. Promoter and enhancer sequences are enriched in SCZ variants associated with eQTL (Roussos et al. 2014). Putative physical interactions between noncontiguous proximal and distal regulatory elements have been identified, and subsequent validation experiments confirmed the existence of a functional loop structure between a distal regulatory element and the gene encoding the L-type calcium channel (CACNA1C). Moreover, this regulatory element overlaps a disease risk locus,

confirming a functional link between SCZ-associated noncoding SNPs, the 3D genome, and the regulation of transcription in the brain. More recently, Won and colleagues generated comprehensive 3D maps of chromatin contacts during human corticogenesis, leading to the identification of hundreds of interactions between genes and distal regulatory elements (Won et al. 2016). Integrating such structural data with SCZ GWAS and eQTL data revealed a number of TF and signaling pathways associated with increased vulnerability to the disease.

Taken together, these studies demonstrate that distal regulatory elements appear to be highly dependent on 3D genomic structures to facilitate their interaction with target genes. Thus, there is a growing body of evidence to suggest that functional disruption of long-range intrachromosomal interactions might contribute to disease, thereby accounting for the observation that the majority of known neuropsychiatric disease risk loci reside within noncoding regions of the genome.

8 Refining the Search to Identify Active Enhancer Elements

All of the aforementioned approaches allow for the genome-wide identification of potential regulatory elements but fail to directly assess their functionality. Techniques such as STARR-seq (*s*elf-*t*ranscribing *a*ctive *r*egulatory *r*egion sequencing) (Arnold et al. 2013; Muerdter et al. 2015), and iterations thereof (Vanhille et al. 2015; Dao et al. 2017), and FIREWACh (*F*unctional *I*dentification of *R*egulatory *E*lements *W*ithin *A*ccessible *Ch*romatin) (Murtha et al. 2014) allow for the identification of active cell-type-specific regulatory elements, 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.

9 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. 3C-based methodologies are a useful tool toward this purpose, as they allow for the identification of physical interactions between distal genetic elements.

The relevance of long-range chromatin interactions to human disease has been examined in a few recent studies. Jager and colleagues used an oligo capture-based approach to Hi-C (capture Hi-C) to identify chromatin interactions of 14 colorectal cancer risk (CRC) loci (Jager et al. 2015). They demonstrated that 3D contacts are

enriched for enhancers and promoters and CRC-specific TF binding sites. The role of long-range chromatin interactions in neurodevelopmental disorders like SCZ has also been investigated (Bharadwaj et al. 2014; Roussos et al. 2014; Won et al. 2016; Zhang et al. 2018).

SNPs associated with SCZ are predominantly located within distal enhancer elements. Integrating Hi-C data with noncoding SCZ GWAS variants revealed that most SNPs interact with non-proximal genes that are specifically involved in pathways related to brain development and function (Won et al. 2016). Many of these long-range chromatin interactions are detected in neuronal cells, are enriched for specific neuronal functions such as axon guidance and synaptic transmission, but are not detected in non-neuronal cells. CRISPR-/Cas9-mediated genome editing in neural progenitors validated the functional relevance of an enhancer harboring a SCZ-associated SNP that interacts with *FOXG1,* a gene encoding a TF implicated in early brain development. Deletion of the region flanking the SNP led to decreased expression of *FOXG1*, but not of the nearby *PRKD1* locus.

The effect of large deletion copy number variants (CNVs) on both the local and global chromatin interactome was investigated in a recent study (Zhang et al. 2018). Zhang and co-workers examined specific changes in chromatin interactions, histone modifications, and gene expression caused by the 3 MB chromosome 22q11.2 deletion, containing more than 60 known genes, which was shown to be associated with several psychiatric disorders, in particular SCZ and autism spectrum disorders. Hi-C analysis on cell lines derived from patients harboring the 22q11.2 deletion CNV showed that intrachromosomal contacts between regions flanking the deletion were strengthened, accompanied by respective changes in H3K27ac and H3K27me3, and associated with increased and decreased transcription, respectively. In addition, there were pronounced changes in the A/B compartment structure and TAD structure spanning the deletion region. There were also changes in inter-chromosomal *trans*-contacts, some of which could be directly attributed to the deletion CNV itself.

10 Concluding Remarks

To better understand these relatively common neuropsychiatric disorders, therefore, requires the systematic study of the regulatory effects of noncoding mutations on gene expression. Toward this end, a number of large collaborative efforts including the CommonMind Consortium (CMC) (Fromer et al. 2016) and PsychENCODE (Akbarian et al. 2015) are underway that focus on the regulatory mechanisms driving gene expression in the human brain. The CommonMind Consortium (www.synapse.org/cmc) aims to generate and analyze large-scale transcriptome data from brain samples, including control specimens and cases with SCZ and BD. The PsychENCODE consortium was established for the purpose of studying the role played by the epigenome in neuropsychiatric diseases (Akbarian et al. 2015). The project primarily focuses on three neuropsychiatric diseases (autism spectrum

disorder, SCZ, and BD). The goal of PsychENCODE is to chart the epigenomic landscape of the brain in a large cohort of cases and controls. This large, collaborative project will employ a variety of methodologies: ChIP-seq will be used to identify putative active promoters and enhancers. In turn, ATACseq will be performed to identify open chromatin regions (Buenrostro et al. 2013), and Hi-C will be used to identify direct interactions between CREs and their downstream target genes (Dekker et al. 2013). The functional relevance of these putative enhancers will be assessed using mouse transgenesis and the STARR-seq assay (Arnold et al. 2013). Importantly, these approaches will be applied to neuronal and non-neuronal nuclei isolated at a number of different developmental time points and from multiple neocortical brain regions. Findings will be validated using neurons derived from induced pluripotent stem cells (iPSCs) and cultured neuronal cells derived from olfactory neuroepithelium (CNON cells).

In summary, there are many exciting findings from recent studies that implicate a clear role for spatial organization of the genome in gene expression regulation, aberrations of which can lead to disease. Much work remains to be done to integrate chromatin organization with gene expression and epigenome data to elucidate the systems biology of neurodevelopmental disorders. However, recent technological advances, including the development of cell-type-specific and single-cell approaches, hold great promise toward identifying the biological pathways and mechanisms that underlie disease.

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DNA Methylation and Hydroxymethylation and Behavior

Sarah R. Rustad, Ligia A. Papale, and Reid S. Alisch

Contents

Abstract Environmentally sensitive molecular mechanisms in the brain, such as DNA methylation, have become a significant focus of neuroscience research because of mounting evidence indicating that they are critical in response to social situations, stress, threats, and behavior. The recent identification of 5-hydroxymethylcytosine (5hmC), which is enriched in the brain (tenfold over peripheral tissues), raises new questions as to the role of this base in mediating epigenetic effects in the brain. The development of genome-wide methods capable of distinguishing 5-methylcytosine (5mC) from 5hmC has revealed that a growing number of behaviors are linked to independent disruptions of 5mC and 5hmC levels, further emphasizing the unique importance of both of these modifications in the brain. Here, we review the recent

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links that indicate DNA methylation (both 5mC and 5hmC) is highly dynamic and that perturbations in this modification may contribute to behaviors related to psychiatric disorders and hold clinical relevance.

Keywords 5-Hydroxymethylcytosine · 5-Methylcytosine · Behavior · DNA methylation · Epigenetics

1 Introduction

The most studied epigenetic modification in the mammalian genome is DNA methylation, which is the addition of a methyl group to the fifth carbon of cytosines (e.g., 5-methylcytosine (5mC)), and is predominantly found at cytosine-phosphate-guanine (CpG) dinucleotide sites (Fig. 1). This DNA modification is catalyzed by DNA methyltransferases (DNMTs), which utilize S-adenosyl-L-methionine as the methyl donor (Cheng 1995). Three active DNMTs have been identified in mammals, each displaying their own distinct functions. DNMT3a and DNMT3b initiate methylation, having an affinity toward unmethylated CpG sites, while DNMT1 preserves methylation, showing preference toward hemimethylated CpG sites (Okano et al. 1999). CpG-rich regions, known as CpG islands, and gene promoters have a significant reduction in 5mC levels, whereas the X chromosome has an overabundance of 5mC (Sharp et al. 2011; Ioshikhes and Zhang 2000). Generally speaking, this DNA modification functions in genomic imprinting, X-chromosome inactivation, chromatin structure, and gene silencing (Bird 2002; Sharma et al. 2010; Han et al. 2015; Suzuki and Bird 2008; Robertson 2005). Human studies support a role for 5mC in the development of behavior-related disorders, including bipolar disorder (BD), schizophrenia (SCZ), and major depressive disorder (MDD), often

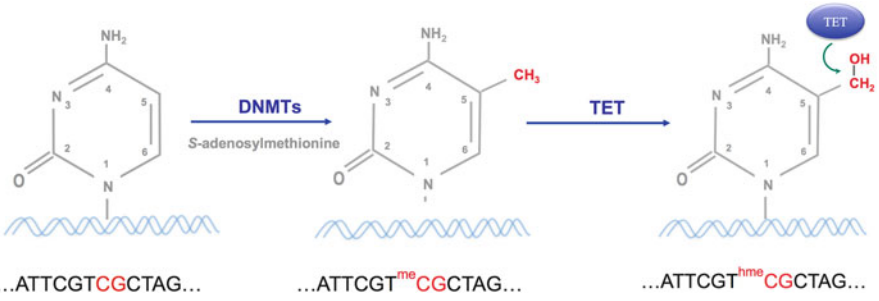

Fig. 1 The process of DNA methylation. Shown is the aromatic ring of cytosine. DNA methyltransferases (DNMTs) and S-adenosylmethionine facilitate the addition of a methyl group (CH_3) to the fifth position of cytosine in a CG (shown in red below; ^{me}CG) dinucleotide context. The ten-eleven translocation (TET) family of enzymes can oxidize the methyl group on cytosine, resulting in hydroxylation (OH; ^{hme}CG). Notably, the TET enzymes can further oxidize the methyl group, resulting in complete demethylation of the cytosine (not shown)

resulting in concomitant changes in gene expression (Abdolmaleky et al. 2006; Poulter et al. 2008; Kuratomi et al. 2008; Kappeler and Meaney 2010; Weaver et al. 2004). It was recently shown that 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC) and that this modification is environmental sensitive (Wu and Zhang 2011), stable (Penn et al. 1972), and highly enriched in the brain (Kriaucionis and Heintz 2009; Sun et al. 2014; Wang et al. 2014; He et al. 2011; Ito et al. 2010; Tahiliani et al. 2009). This oxidation process is catalyzed by the ten-eleven translocation (TET) family of enzymes and can contribute to active demethylation, whereby two mechanisms can convert 5hmC back into cytosine; iterative oxidation by TET enzymes, which continuously oxidize 5hmC; and deamination by activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex AID/APOBEC (Ito et al. 2011; Guo et al. 2014).

While the full functional potential of 5hmC is yet to be determined, data supporting several putative molecular mechanisms have been found, including the regulation of transcription factor (TF) binding (Li et al. 2016), sex-specific development (Gross et al. 2015), and transcript diversity through interactions with the spliceosome (Feng et al. 2015) (Fig. 2). It is noteworthy that traditional DNA methylation detection methods utilizing sodium bisulfite treatment cannot distinguish between the methylated and hydroxymethylated forms of cytosine, meaning that past studies using such methods report a composite of 5mC and 5hmC but have attributed any findings solely to 5mC. This chapter will provide evidence to support the independent importance of 5mC and 5hmC in mental health and the development of mental illness and present several putative molecular functions of DNA methylation that may shed light on its promising clinical relevance. With recent findings implicating dynamic and unique roles for 5mC and 5hmC in behavior, DNA methylation brings a new frontier to the field of psychiatry.

2 Total DNA Methylation (5mC + 5hmC) and Behavior

A growing body of evidence suggests that the methylome varies dramatically throughout life, as the result of extrinsic influences such as environmental and physical factors (Tammen et al. 2013; King-Batoon et al. 2008; Park et al. 2012). One such factor with robust connections to DNA methylation is psychological adversity though most of these links were found using animal models. For example, rodent mothers that performed enhanced licking, grooming, and arched back nursing altered their offspring's DNA methylation levels on the glucocorticoid receptor (*NR3C1*) gene in the hippocampus, which is a gene best known for its role in the stress response. These mother-to-offspring interactions affected the development of their offspring's hypothalamic-pituitary-adrenal (HPA) axis, which is a dynamic metabolic system that regulates homeostatic mechanisms, including future reactions to new environments. Similarly, an abusive caregiver leads to lifelong increases in offspring DNA methylation on the brain-derived neurotrophic factor (*Bdnf*) gene in the prefrontal cortex of both male and female rodents, which can persist to the next

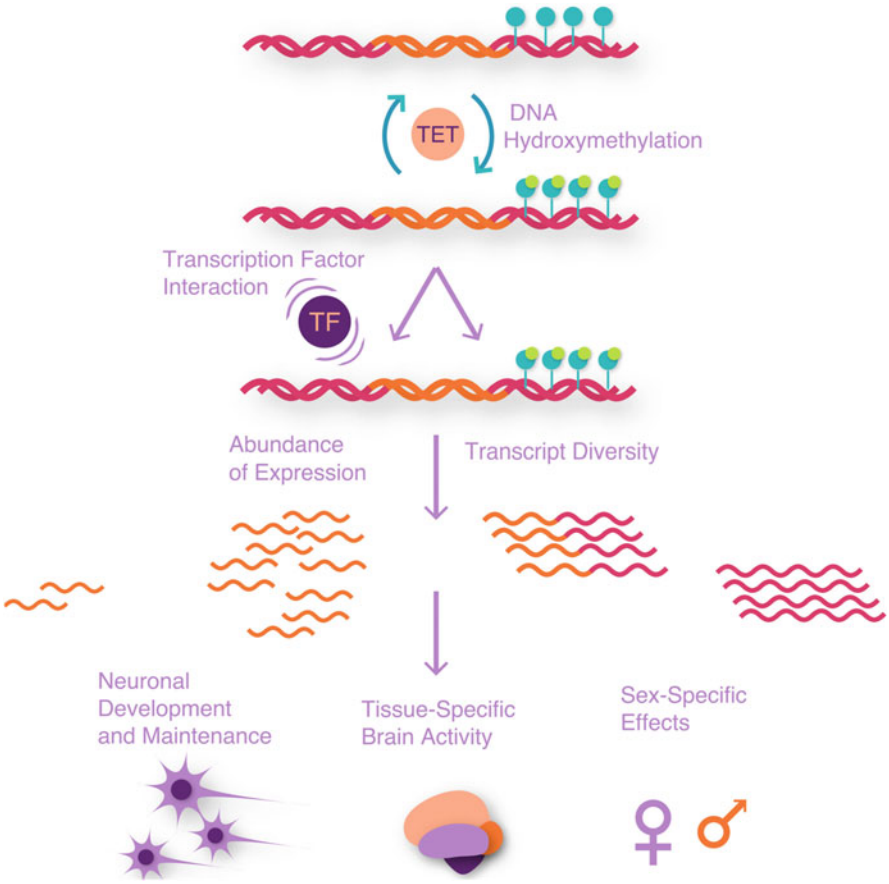

Fig. 2 Putative molecular mechanisms of 5hmC. Following oxidation by the TET enzymes, the 5hmC modification has been shown to interact with transcription factors (TF) and affect transcript abundance. In addition, 5hmC modifications have been implemented in the splicing process, resulting in transcript diversity. Ultimately, these changes in transcript expression and diversity can have lasting effects at the cellular and tissue level, in a sex-specific manner

generation (Roth et al. 2009). More recent work also found increased DNA methylation on *Bdnf* and concomitant decreased expression in the amygdala and hippocampus of rats exposed to prenatal stress (Boersma et al. 2014). Studies in young monkeys identified differentially methylated genes that are implicated as risk factors for developing anxiety and depressive disorders (Alisch et al. 2014). In addition, several other studies have shown evidence of altered DNA methylation and gene expression due to maternal separation or varied maternal care and prenatal stress (Beery et al. 2016; Murgatroyd et al. 2009; Mueller and Bale 2008; Novikova et al. 2008).

Human studies are obviously more difficult to conduct, due to limited access to brain tissue, making them slow to contribute to these findings. Nonetheless, more

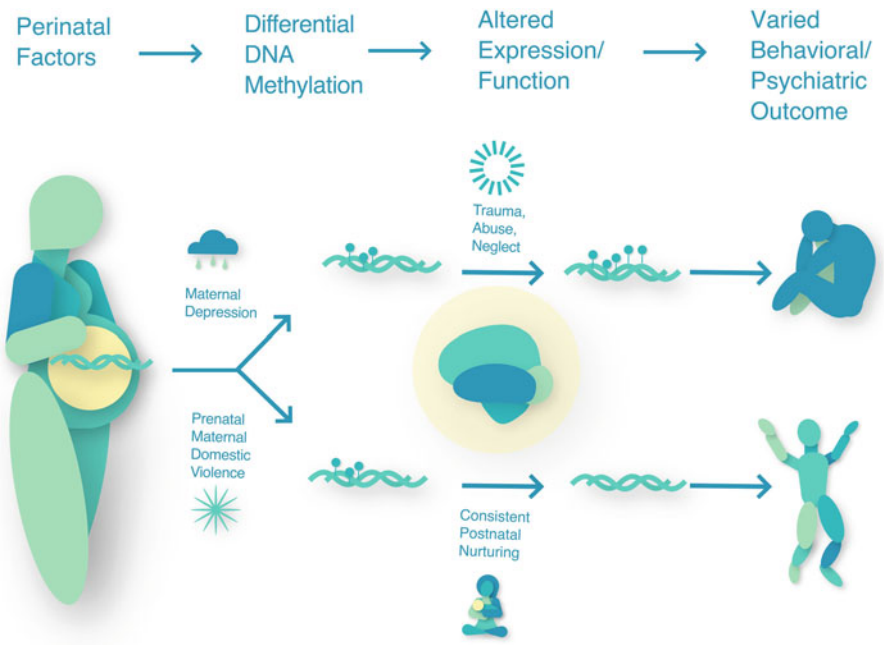

Fig. 3 Perinatal environmental adversities impact DNA methylation levels linked to human behavior. Perinatal exposure to maternal depression and negative experiences can alter DNA methylation in the perinatal brain, resulting in psychiatric disorders later in life. However, postnatal nurturing may reverse these effects and lead to healthier psychiatric outcomes

recent human studies also have shown that stress, anxiety, depression, neglect, socioeconomic status, violence, and maternal care all impact DNA methylation levels (Fig. 3) (Kim et al. 2016; Lam et al. 2012; King et al. 2015; Naumova et al. 2012), for example, suicide completers who were exposed to childhood abuse and neglect carried hypermethylation in the promoter regions of ribosomal RNAs and concomitant downregulated expression in the hippocampus (Mcgowan et al. 2008). However, aberrant DNA methylation patterns due to psychological influences were not restricted to promoter regions, as they also were linked to changes in gene bodies, intergenic regions, and 5′ and 3′ untranslated regions (UTRs) (Weder et al. 2014). Moreover, stress-dependent DNA methylation levels can be linked to genotype-specific gene transcription, resulting in a long-term dysregulation of the stress hormone system (Klengel et al. 2013). Together, these studies suggest that early-life adversities increase the risk of behavioral disturbances into adulthood and that these outcomes are governed by DNA methylation. Evidence of the environmental impact on DNA methylation patterns is often examined in the HPA axis. We start with *NR3C1*, the human glucocorticoid receptor gene, which is highly abundant in the HPA system.

2.1 The Perinatal Period

To examine the effects of offspring prenatal exposure to maternal psychological well-being on DNA methylation status at the promoter and exon 1F of the human *NR3C1* gene, cord blood from 82 newborn girls was collected, and maternal mood was measured using the Hamilton Rating Scale for Depression (HAM-D), Hamilton Rating Scale for Anxiety (HAM-A), and Edinburgh Postnatal Depression Scale (EPDS) (Oberlander et al. 2008). Increased scores in the third trimester for all three tests positively correlated with the DNA methylation levels on *NR3C1*, including sites containing the NGIF-A (transcription factor) binding site in exon 1F of *NR3C1*. Interestingly, saliva was collected from the offspring at 3 months of age, before and after exposure to negative visual stimuli, to measure cortisol stress response. Infants who responded with high cortisol levels had considerably higher *NR3C1* DNA methylation levels when compared with infants whose cortisol levels decreased in response to the stress assessment. These data suggest that maternal depression in the third trimester directly impacts DNA methylation on the *NR3C1* gene and alters the infant's stress response. Another study similarly found increased whole blood DNA methylation levels on exon 1F of *NR3C1* only in the offspring (Radtke et al. 2011). Notably, the tested offspring were between 10 and 19 years of age, indicating that exposure to maternal anxiety and stress in the intrauterine environment induced stable disruptions in DNA methylation. Similar to the licking and grooming studies conducted in rodents, maternal stroking in human infants leads to fluctuations of methylation of the *NR3C1* gene (Murgatroyd et al. 2015). In this study, DNA methylation levels were examined in saliva samples from 181 infants and showed that high maternal stroking of the infant face, back, abdomen, legs, and arms reduced *NR3C1* DNA methylation levels. This study highlighted the extreme vulnerability and sensitivity of the early postnatal period to extrinsic factors that may directly impact fetal development. While several groups have found links between maternal emotion, stress, childhood adversity, and the methylation status of exon 1F in the promoter of *NR3C1* (Mulligan et al. 2012; Hompes et al. 2013; Radtke et al. 2015; Braithwaite et al. 2015), sex-specific differences in *NR3C1* DNA methylation levels also have been shown, as male infants specifically exhibited elevated exon 1F methylation due to maternal depression during pregnancy (Braithwaite et al. 2015). This finding may shed mechanistic light on sex-specific behaviors, including psychological disorders.

Studies of extrinsic factors impacting development during the perinatal period also have focused on the neurotransmitter serotonin (*5-HT* or *SLC6A4*), which is linked with impulsive aggression as well as increased susceptibility for a lifetime risk of depression (Devlin et al. 2010; Feinn et al. 2005; Seo et al. 2008). A study of prenatal maternal depression in 82 pregnant women found that second trimester DNA methylation levels were lower on the *SLC6A4* promoter in mothers with higher depressed mood symptoms (Devlin et al. 2010). These decreased DNA methylation levels likely result in increased expression of *SLC6A4*, higher serotonin uptake, and decreased intrasynaptic serotonin, though such studies have not yet been conducted.

However, as serotonin modulates neuronal differentiation and growth, altered levels of the serotonin transporter during critical developmental periods likely affect brain development and may have long-term effects on subsequent child emotional development and susceptibility to affective disorders later in life (Ansorge et al. 2004, 2008; Gaspar et al. 2003).

Another mediator of neural function and plasticity is the brain-derived neurotrophic factor (*BDNF*), which is essential for neurogenesis and has been associated with a variety of affective disorders later in life (Mcewen 2007; Roth and Sweatt 2011a; Berton et al. 2006; Fuchikami et al. 2011; Keller et al. 2010; Post 2007). The genetic structure of the *BDNF* gene is complex, suggesting an intricate regulatory system. The gene consists of nine 5′ noncoding exons that are each linked to a distinct promoter that controls differential expression of exon-specific transcripts (Roth et al. 2009; Aid et al. 2007; Liu et al. 2006). Examination of *BDNF* DNA methylation levels related to prenatal maternal depression in 57 infants found reduced DNA methylation on the *BDNF* exon IV promoter. Interestingly, the reduced DNA methylation levels were located near the binding site of CREB, a transcription factor that regulates *BDNF* transcription via a DNA methylation-dependent mechanism, suggesting that prenatal maternal depression may affect this mechanism.

Oxytocin is a neuropeptide hormone that regulates reproductive physiology and contributes to maternal behaviors, pair bonding, and social interaction (Pedersen et al. 2006; Young and Wang 2004; Carter 2003; Winslow and Insel 2002; Carter et al. 1992; Pedersen and Boccia 2002; Popik and Van Ree 1991). An investigation into the potential interplay between oxytocin, DNA methylation, and the development of psychopathy examined the oxytocin receptor (*OXTR*) gene in blood from male children aged 4–16 years that had severe ratings of child conduct problems, as measured using the clinician rating scale called the Quality of Family Environment (Dadds et al. 2014; Rey et al. 1997). Interestingly, younger children (4–8 years) did not exhibit conduct-related DNA methylation levels on the *OXTR* gene; however, older children (9–16 years) had a positive correlation between conduct problems and DNA methylation levels on *OXTR*. This increased methylation in older children also had a direct impact on the level of circulating oxytocin, suggesting impairment of the entire oxytocin system, highlighted by the methylation-dependent downregulation of *OXTR* expression (Dadds et al. 2012, 2014). Indeed, childhood stress is linked to differential methylation of oxytocin (*OXT*) in the saliva of older girls (10–12 years) (Papale et al. 2018). Interestingly, a related study found that increased DNA methylation on the *OXTR* gene, and subsequent reduced expression, in the temporal cortex served as a valid indicator of autism spectrum disorder (ASD) (Gregory et al. 2009), differentiating between ASD and control subjects. Clearly the abundance of DNA methylation can be used to assess social behaviors in children.

While these aforementioned studies and many others were narrowly focused on a single promoter and gene, the use of genome-wide approaches has greatly improved our understanding of the impact of extrinsic factors on child susceptibility to affective disorders. One study collected saliva from 94 maltreated children and 96 controls (all 5–14 years of age) and found trauma-associated DNA methylation

levels across the genome, including some on well-known stress-related genes, such as *NR3C1*, *BDNF*, and FK506 binding protein 51 (*FKBP5*), as well as on biologically relevant genes, including *ID3* (DNA-binding protein inhibitor ID-3), *TPPP* (tubulin polymerization promoting protein), and *GRIN1* (glutamate (NMDA) receptor NR1 subunit) (Weder et al. 2014). Moreover, since *ID3* expression is increased following stress (Konishi et al. 2010), the variation in salivary cortisol can predict *ID3* methylation-related diurnal cortisol secretion. Another study that examined saliva from 11 girls that experienced extremely high levels and 11 girls with normative levels of early childhood stress exposure (all 9–12 years of age) also found trauma-related DNA methylation levels across the genome at more than 100 genes, including on stress-related genes like *OXT* and a serotonin receptor: *HTR3A* (Papale et al. 2018). Together, these data suggest that DNA methylation is stable long after trauma exposure and may hold predictive information of mental well-being and prognosis.

2.2 The Adult Period

Variations in DNA methylation levels due to early-life adversity can persist into adulthood. Studies have linked altered DNA methylation levels and gene expression of hippocampal *NR3C1* and psychological illnesses, including mood disorders and SCZ, which are associated with suicide in adults; indeed, often suicide relates to a history of childhood abuse (Heim and Nemeroff 2001; Webster et al. 2002). In one such study, postmortem hippocampal samples were obtained from suicide completers that had traumatic childhoods, suicide completers that did not have a history of abuse, and controls that had sudden accidental deaths and did not experience abuse (Mcgowan et al. 2009). The suicide completers who had traumatic childhoods showed higher DNA methylation in exon 1F of *NR3C1*, compared to both controls (i.e., suicide completers without a history of abuse and victims of sudden accidental death). As predicted, the increased DNA methylation levels interrupted NGIF-A binding to *NR3C1* and gene transcription.

Suicide victims also show decreased *BDNF* expression levels in the hippocampus and prefrontal cortex, suggesting that *BDNF* plays a critical role in pathophysiological aspects of suicidal tendencies. Examination of *BDNF* DNA methylation levels in postmortem brain samples (Wernicke's area of the brain) from 44 suicide completers and 33 controls revealed hypermethylation in suicide completers, which was shown in vitro to result in lower *BDNF* mRNA levels (Keller et al. 2010). This study showed that a gene-specific increase of DNA methylation downregulates its expression, which can account for the pathophysiology observed the suicidal brain.

Another study examined the blood of 101 women aged 30 and 41 years that had borderline personality disorder (BPD) and had experienced extreme childhood sexual, physical, and emotional traumas; 99 of the women had MDD and 15 had MDD and post-traumatic stress disorder (PTSD) (Perroud et al. 2011). A positive correlation was found between sexual abuse severity and *NR3C1* DNA methylation

levels. Similarly, physically and/or emotionally abused and neglected participants all had higher *NR3C1* DNA methylation than controls. In a related study, blood samples from 115 patients with BPD and 52 controls were collected for DNA methylation analysis, and the patients were found to exhibit higher *BDNF* DNA methylation compared to controls, which was positively correlated with childhood trauma such as sexual, physical, and emotional abuse and physical and emotional neglect (Perroud et al. 2013). Similarly, a positive association was observed between *BDNF* DNA methylation levels and hopelessness, depression severity, and impulsivity. Finding higher *BDNF* DNA methylation in BPD patients is consistent with the higher *BDNF* DNA methylation of suicide completers, and suicidal tendencies are common in BPD patients. Interestingly, these patients were subjected to intensive dialectical behavior therapy, which reduced *BDNF* DNA methylation and BPD symptoms in responding patients. While a similar increase in *BDNF* DNA methylation was observed in BD patients compared to controls, it was hypothesized that therapies such as antidepressants contributed to these elevated levels of *BDNF* DNA methylation. Together, these data suggested that *BDNF* DNA methylation levels might be a biomarker of BD therapy efficacy.

Childhood abuse also can stably alter DNA methylation levels that may contribute to the development of adult eating disorders, which are often comorbid with personality disorders. Examination of blood from 32 women with bulimia nervosa that had a history of childhood abuse, 32 women with bulimia nervosa that did not have a history of childhood abuse, and 32 controls revealed higher DNA methylation in exon 1C of *NR3C1* in women with bulimia nervosa and history of abuse compared to both controls (Steiger et al. 2013). While these findings do not definitively link DNA methylation to bulimia nervosa, they do suggest that the dysregulation of HPA activity, which contributes to the predisposition to psychological disorders observed in adults, originates early in life and is marked by stable changes in DNA methylation that are correlated to abuse severity. Other reports have corroborated these findings (Lawson et al. 2012; Maguire et al. 2013; Frieling et al. 2008; Toyokawa et al. 2012). In one such study, buccal cells from 15 anorexia nervosa patients and 36 controls were examined for DNA methylation levels on the *OXTR* gene (Kim et al. 2014). All participants were assessed using the Eating Disorder Examination Questionnaire (EDE-Q) (Fairburn and Beglin 1994), the Beck Depression Inventory (BDI), the Spielberger State and Trait Anxiety Inventory (STAI) (Speilberger et al. 1983), and the Autism Spectrum Quotient (ASQ) (Baron-Cohen et al. 2001), since eating disorders and autism have common traits including focus on detail, rigidity, and social cognition. *OXTR* DNA methylation levels were positively correlated with dietary restraint (EDE-Q), communication (ASQ), and depression and anxiety (BDI and STAI) and inversely correlated with body mass index (BMI). Notably, a linear regression of these data revealed that eating disorder psychopathology, BMI, and anxiety were the main determinants of the differential *OXTR* DNA methylation levels found in patients compared to controls. While it is not known whether DNA methylation levels in buccal cells truly reflect the levels in the brain, this study provides insight into a role for DNA methylation in eating disorder psychopathology.

One potential way to further link peripheral DNA methylation to brain function is by pairing DNA methylation data with functional magnetic resonance imaging (fMRI) data. One such study took this approach and showed that adverse childhood experiences can have long-lasting effects on DNA methylation-associated brain activity. This relationship was demonstrated in 25 mothers with interpersonal violence (IPV)-related PTSD, 9 with PTSD symptoms (non-IPV related), and 20 controls, revealing a positive correlation between *BDNF* DNA methylation and maternal brain activity in the ventromedial prefrontal cortex and anterior cingulate, which are regions associated with emotion regulation (Moser et al. 2015; Hu and Jiang 2014). Interestingly, there was a negative correlation in the right hippocampus and parahippocampus, left and right precuneus, left cerebellum, and right superior temporal gyrus. Together, these findings suggest that long-lasting changes in peripheral *BDNF* DNA methylation levels may reflect disrupted functions in many regions of the brain.

Using a similar approach, blood was collected from 42 participants that were subjected to social perception tasks during fMRI. These tasks recruit a network of brain structures that are vital to social perception and mentalizing abilities, such as the temporal parietal junction. A positive correlation was observed between *OXTR* DNA methylation levels and brain activity in the temporal parietal junction and dorsal anterior cingulate cortex (Jack et al. 2012). Moreover, the degree to which *OXTR* DNA methylation fluctuated was positively associated with BOLD activity (blood oxygenation level-dependent signal, which reflects neural activity) from the superior temporal gyrus into the supramarginal gyrus at the temporal parietal junction and the dorsal anterior cingulate cortex. The dorsal anterior cingulate cortex plays a central role in social and affective appraisals of motivationally salient stimuli. Functional impairment of this region is linked to emotional and social deficits in anxiety disorders and ASD (Etkin et al. 2011; Mcclure et al. 2007). Others also have shown that increased *OXTR* DNA methylation levels were concomitant with increased activity in the amygdala and high neural response, when observing negative facial expressions. Together, these studies highlighted that emotional and social perceptual processes involving the oxytocin system may be governed by DNA methylation.

Of course variations in DNA methylation also have been associated with psychological conditions in males, including anxiety, depression, hostility, happiness, and general life satisfaction. Examination of DNA methylation in blood of more than 500 men older than 70 years of age revealed psychological-related relationships to DNA methylation levels in several promoter regions of genes involved in immune/inflammatory processes related to atherosclerosis. For example, increased DNA methylation at the *ICAM-1* promoter was associated with increased anxiety, depression, and hostility, whereas happiness and life satisfaction showed an inverse correlation with DNA methylation. Notably, while higher promoter DNA methylation is expected to decrease *ICAM-1* expression, these psychological outcomes were not associated with lower serum *ICAM-1* levels. At the *TLR-2* promoter, DNA methylation levels were positively associated with hostility, while higher life satisfaction led to lower DNA methylation levels. At the coagulation factor III (*F3*)

promoter, DNA methylation levels were positively correlated with depression, whereas life satisfaction and happiness were inversely associated with *F3* DNA methylation. Finally, at the iNOS promoter, DNA methylation levels were inversely correlated with depression and anxiety, but happiness and life satisfaction positively correlated with DNA methylation. Notably, significant correlations were not found at the *NR3C1* promoter, which is in contrast with several other studies that have found associations with negative psychological factors and DNA methylation on the *NR3C1* promoter (Braithwaite et al. 2015; Perroud et al. 2011). This was one of the very few studies that associated an optimistic psychological factor (happiness) with DNA methylation levels, demonstrating a role for DNA methylation across the psychological spectrum.

To examine DNA methylation levels on the serotonin transporter gene (*SLC6A4*) in adults related to childhood aggressive behaviors, blood was collected from 25 healthy adult males (mean age of 27 years). Here, a positive correlation was found between childhood aggression and DNA methylation in monocytes and T cells (Wang et al. 2012a). This study also used positron emission tomography (PET) measures of brain serotonin synthesis and found inverse associations between mean *SLC6A4* DNA methylation and *SLC6A4* expression in the lateral left and right orbitofrontal cortex. Notably, a specific *SLC6A4* genotype, which is known to affect gene expression, was not correlated to DNA methylation levels. On the other hand, in vitro analysis confirmed that the aggressiveness-related DNA methylation levels decreased *SLC6A4* transcriptional activity, suggesting that peripheral DNA methylation might be used to screen for serotonin-related psychological and psychiatric disorders and allow for preventive and corrective interventions. A subsequent study found that more severe physical childhood abuse along with reduced hippocampal volume was associated with higher DNA methylation on the *SLC6A4* promoter (Booij et al. 2015). This finding is consistent with the fact that the hippocampus is richly innervated with serotonin and is central in regulation of stress (Frodl and O'Keane 2013). DNA methylation on the *SLC6A4* promoter also has been studied in adult monozygotic twins that are discordant for MDD or PTSD (Zhao et al. 2013). Here genomic DNA was extracted from blood leukocytes of 84 monozygotic twin pairs (168 total participants), revealing that increased *SLC6A4* promoter DNA methylation was associated with depressive symptoms. Finding associations between childhood abuse, peripheral *SLC6A4* DNA methylation, brain *SLC6A4* expression, and hippocampal volumes further supports that peripheral DNA methylation levels may serve as a valuable biomarker for serotonin-associated stress-related psychopathology.

Social anxiety disorder (SAD) is characterized by fear, anxiety, and evasion of social situations for fear of being negatively evaluated or scrutinized and rejected (Labuschagne et al. 2010; Stein and Stein 2008). To characterize the role of DNA methylation in SAD patients, blood was collected from 110 patients and 110 age- and sex-matched controls. The severity of social anxiety in both patients and controls were assessed using the Social Interaction Anxiety Scale and Social Phobia Scale (SIAS and SPS) (Stangier et al. 1999). Analysis of the *OXTR* promoter revealed significantly lower DNA methylation compared to controls and a negative

correlation between *OXTR* DNA methylation levels and SIAS and SPS scores (Ziegler et al. 2015). This finding was corroborated in independent saliva samples from 16 healthy females subjected to the Trier Social Stress Test that showed a negative correlation between *OXTR* DNA methylation and maximum salivary cortisol. An additional test on SAD patients using fMRI revealed increased amygdala activation and decreased *OXTR* DNA methylation during social phobia-related word processing. Together, this detailed study found that decreased *OXTR* DNA methylation was associated with SAD, stress-related cortisol levels, and heightened amygdala response, revealing the extensive role of DNA methylation in this chronic mental health condition.

Obsessive-compulsive disorder (OCD) is a severe disorder characterized by troubling thoughts and obsessions, such as the need for symmetry, exactness, and order and contamination fears and worries about harm to self and others (Barrett and Healy 2003; Lack 2012; Swedo et al. 1989). To investigate a role for DNA methylation in OCD, genomic DNA was obtained from the blood of 42 OCD patients that had Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) scores of $\geq$16 for the combination of compulsions and obsessions or $\geq$10 for compulsions and obsessions alone and 31 controls (all 18–65 years old) (Cappi et al. 2016; Goodman et al. 1989). *OXTR* DNA methylation levels were higher in OCD patients compared to controls and negatively correlated with depression severity (Cappi et al. 2016); this relation to depression contrasts the findings of other studies (Kim et al. 2014; Ziegler et al. 2015). It is unknown whether the OCD-related hypermethylation of *OXTR* influences *OXTR* expression. A similar study of psychosis recruited 167 men and women with a psychotic disorder, including SCZ, BD, and schizoaffective disorder, and 75 controls (Rubin et al. 2016). Here, females displayed higher *OXTR* DNA methylation that was linked to poorer recognition of emotional expressions. Moreover, while a positive association between *OXTR* DNA methylation levels and oxytocin levels were found in females, a negative association was found in males. Together, these sex-specific differences in *OXTR* DNA methylation and oxytocin levels may hold key information into more personalized therapeutic treatment of SCZ and BD.

Studies of DNA methylation levels in novel genes related to psychological conditions are being reported on a continual basis. For example, DNA methylation levels in the promoters of *ZNF266*, *AGTR1*, *ASPH*, *PLAC1L*, and numerous other genes have been linked to chronic physical aggression in both males and females (Guillemin et al. 2014; Provencal et al. 2013). SCZ patients exhibit differential DNA methylation in the first intron of *RELN* (Aberg et al. 2014) and in several glutamate receptor genes (*GRIA2*, *GRIA3*, *GMR2*, *GMR5*, *GMR8*) (Aberg et al. 2014; Kordi-Tamandani et al. 2013) when compared to controls. In addition, BD patients have higher DNA methylation in *PRIMA1* compared to controls. It is of great interest to confirm the role of the aberrant DNA methylation levels of these genes and determine if they can be used for novel diagnostic, prognostic, and modifiable therapeutic targets.

2.3 In the Pathogenesis of Neurodegeneration

Dynamic changes in DNA methylation levels have been linked with aging and memory loss, leading to neurodegeneration (Johnson et al. 2012; Day and Sweatt 2010; Day et al. 2013; Sanchez-Mut et al. 2016; Alisch et al. 2012). Indeed, several groups have shown locus-specific and/or global disruptions of DNA methylation abundance in postmortem brain tissue of Alzheimer's disease (AD) patients, though findings differ between studies, suggesting a complex involvement of DNA methylation in AD pathogenesis (Bakulski et al. 2012; Lunnon et al. 2014; De Jager et al. 2014; Watson et al. 2016; Coppieters et al. 2014). One such study isolated genomic DNA from four postmortem brain regions of 122 AD patients and premortem whole blood when available ($n = 57$) and measured genome-wide DNA methylation levels associated with Braak staging, a standardized measure of neurofibrillary tangle burden determined at autopsy (Braak and Braak 1991). This approach revealed that the DNA methylation levels in the ankyrin 1 (*ANK1*) gene were associated with neuropathology in the entorhinal cortex, superior temporal gyrus, and prefrontal cortex, but not in the cerebellum or whole blood of the same AD patients. These data suggest that AD-associated variation in DNA methylation is consistent across pathologically relevant regions of the brain. In addition, recent epigenome-wide association studies (EWAS) detected both cross-tissue and tissue-specific DNA methylation profiles in brain tissue, identifying differential methylation in candidate genes of AD and in genes previously unassociated with AD (Bakulski et al. 2012; Lunnon et al. 2014; De Jager et al. 2014; Watson et al. 2016). Together, these studies underscore the utility of EWAS in identifying novel genes and pathways associated with AD pathogenesis that may otherwise be overlooked. Notably, however, these previous studies have relied heavily on the accessibility of brain tissue. Shifting studies of DNA methylation to peripheral whole blood provides a potential tool that may be utilized clinically to improve diagnosis and guide personalized treatment of AD. Support for this approach came from a recent study that extracted genomic DNA from the whole blood of 45 late-onset AD (LOAD) patients and 39 matched controls and found differentially methylated positions (DMPs) that distinguish people with and without LOAD (Madrid et al. 2018). Interestingly, subsequent independent comparison using six continuous clinical LOAD phenotypes as variables, comprising RAVLT scores, and CSF t-tau and p-tau$_{181}$ levels, or t-tau/Aβ_{42}, p-tau$_{181}$/Aβ_{42}, or Aβ_{42}/Aβ_{40} ratios, yielded a unique set of 17 DMPs that were all hypomethylated in two genes, *B3GALT4* (beta-1,3-galatcosyltransferase 4) and *ZADH2* (prostaglandin reductase 3). Taken together, these data reinforce the use of blood as an accessible tissue of value in the identification of DMPs associated with dementia onset and progression.

Parkinson's disease (PD) is the second most common chronic neurodegenerative disease in the elderly population. While the motor-related symptoms that characterize PD are bradykinesia, tremor, rigidity, and postural instability, the behavioral deficits include depression, anxiety, sleep disorders, and cognitive dysfunction. Collectively, these outcomes lead to severe impairment of the quality of life for

PD patients (Frucht 2004). The first genetic cause of PD was identified as a missense mutation in the alpha-synuclein (*SNCA*) gene (SNCAp.Ala53Thr), a locus that also was found to undergo duplications and triplications linked to PD (Polymeropoulos et al. 1997). *SNCA* gene dosage is critical for the development of PD, leading researcher to hypothesize that deregulation of *SNCA* may be a potential mechanism for PD. This hypothesis was confirmed when DNA methylation levels of *SNCA* were found to be reduced in the substantia nigra, putamen, and cortex of PD patients ($N = 12$) compared to controls ($N = 14$) and was linked to the increased expression of *SNCA* (Jowaed et al. 2010). In 2011, a comprehensive genomic study identified several PD risk loci in cerebellum and frontal cortex of PD brains, including *PARK16*, *GPNMB*, and *STX1B* genes, all of which were associated with differential DNA methylation at proximal CpG sites (International Parkinson's Disease Genomics C and Wellcome Trust Case Control C 2011). Examination of DNA methylation levels across the genome in the frontal cortex and blood leukocytes from the same PD patients ($N = 5$) and controls ($N = 6$) found a high concordance of genes differentially methylated in both tissues (Masliah et al. 2013). Taken together, these data reinforce the use of blood as an accessible tissue of value in the identification of differentially methylated sites associated with PD onset and progression and lend further support to the pathogenesis of PD, by providing novel diagnostic, prognostic, and modifiable therapeutic targets.

Huntington's disease (HD) is characterized by deficits in cognitive, psychiatric, and motor stability that is typically caused by a trinucleotide repeat (CAG) mutation in the *HTT* gene (Walker 2007). Expression of the disease protein, huntingtin, leads to extensive transcriptional dysregulation, and a growing body of evidence suggests that epigenetic modifications play a key role in HD pathogenesis (Lee et al. 2013; Reik et al. 1993). Although links between DNA methylation and HD are only beginning to emerge, several recent studies have reported HD-related changes in DNA methylation in rodents and humans. One such study targeted the *ADORA2A* gene, a G-protein-coupled receptor that decreases its expression in HD patients, and found increased levels of DNA methylation in the putamen brain tissue of HD patients ($N = 6$) compared to controls ($N = 5$) (Villar-Menendez et al. 2013). Interestingly, contradictory results were found when genome-wide approaches were employed to identify HD-related changes in DNA methylation throughout the genome. While one study found thousands of differentially methylated sites in a fibroblast cell line from an HD patient compared to control, another study failed to find differentially methylated sites in genomic DNA from the forebrain cortex brain tissue from seven HD patients and six controls (Jia et al. 2015; De Souza et al. 2016). Of course this latter study was more complex, profiling multiple cell types from both sexes, and the data was stringently normalized to account for this complexity, which may contribute for the lack of findings. Nonetheless, studies profiling rodent brain tissues have clearly shown that aberrant DNA methylation levels are associated with HD pathogenesis (Ng et al. 2013), indicating that larger human sample sizes are needed to definitely determine the role of DNA methylation in HD.

Finally, it was recently shown that patterns of DNA methylation correlate with chronologic age with high precision, thereby providing a "DNA methylation age"

(Horvath 2013); importantly, the gap between chronologic age and DNA methylation age widens with environmental exposures and clinical disease (Almen et al. 2014; Kananen et al. 2015; Beach et al. 2015). An accelerated DNA methylation age – that is, when estimated age is higher than expected (on the basis of chronological age) – predicts several age-related cognitive phenotypes. Notably, accelerated DNA methylation age has been associated with Alzheimer's, Parkinson's, and Huntington's disease (Horvath et al. 2016; Levine et al. 2015; Horvath and Ritz 2015; Chen et al. 2016). While it is currently unclear what these biomarkers can teach us about the biology of these disorders, their longitudinal investigation could help determine the impact of endogenous or exogenous stress factors on disease onset and progression. Perhaps the most exciting feature of identifying DNA methylation biomarkers is that epigenetic changes are reversible, raising the prospect that DNA methylation age estimates might be useful for identifying or validating interventions.

3 5hmC and Behavior

The elucidation of the link between 5hmC and behavior is still in its infancy, especially in humans. Aside from its relatively recent discovery in mammalian cells in 2009 (Kriaucionis and Heintz 2009; Tahiliani et al. 2009), the primary reason for this delay in humans, as compared to 5mC, is that 5hmC has a very low abundance in peripheral tissue, making access to brain tissue paramount for these studies. Nonetheless, in less than a decade, numerous studies in mice have led to the emergence of a putative role for 5hmC in behaviors related to psychiatric disorders. Accordingly, this section will feature more findings from rodent studies than humans, simply due to the limited number of human studies performed to date.

Studies have demonstrated an age-dependent accumulation of 5hmC in the brain throughout early-life development and into adulthood (Szulwach et al. 2011; Chen et al. 2012; Zampieri et al. 2015). Environmental stimuli can alter this age-associated accumulation. For example, calorically restricted mice exhibited an age-dependent reduction of 5hmC levels in hippocampal and cerebellar tissue (Chouliaras et al. 2012). In addition, mice exposed to an enriched environment had reduced 5hmC abundance in the hippocampus, primarily on genes involved in axon guidance (Irier et al. 2014). These alterations also were associated with increased learning and memory, suggesting that environmental enrichment might modulate the dynamics of 5hmC in the hippocampus and contribute to improved learning and memory. In contrast, mice subjected to a 30-min acute stress showed increased 5hmC levels on the glucocorticoid receptor gene (*Nr3c1*) in the hippocampus (Li et al. 2015). Genome-wide 5hmC analysis of these same mice revealed that short-term stress induced genome-wide disruptions of 5hmC and confirmed an overall increase in 5hmC following stress. Interestingly, altered 5hmC was found near several transcription factor (TF) binding sites of genes that were differentially expressed and have known roles in neurogenesis and neurological activities (Li et al. 2016),

suggesting that in response to stress, the function of 5hmC may be to influence TF binding to provide appropriate levels of gene expression needed to cope with the stress. The fact that 5hmC changes were found within 1 h of a short stress highlights the potential for rapid changes of 5hmC within the brain. It will be interesting to examine the long-term effects of short stress (i.e., more than 1 h after exposure) or how chronic stress alters 5hmC levels. Many believe 5hmC to be important in long-term consequences of mental health, yet these studies indicate that alterations in 5hmC can occur rapidly and may impact the expression of key genes related to the origins of mental illness.

Environmental stimuli can affect brain regions other than the hippocampus. For example, mice exposed to repeated administrations of cocaine have increased 5hmC in the nucleus accumbens, primarily in coding regions and enhancer sequences of genes involved in drug addiction (Feng et al. 2015). Notably, 5hmC changes persisted for a minimum of 1 month after cocaine exposure in only a small subset of loci, suggesting that these epigenetic changes are largely reversible. The modulation of 5hmC also may mediate behavioral adaptations. For example, fear extinction, a form of reverse learning, results in dramatic 5hmC changes in the prefrontal cortex of mice (Li et al. 2014). These studies also support unique molecular roles for the *Tet* enzymes, as *Tet3*, but not *Tet1*, mediated the increased gene expression that was associated with rapid behavioral adaptation. Interestingly, another group found that mice lacking the expression of *Tet1* exhibited impaired memory extinction, coupled with long-term synaptic depression and downregulation of neuronal activity-related genes (Rudenko et al. 2013). Thus, while *Tet3* may solely facilitate the accumulation of 5hmC in the prefrontal cortex of mice during rapid behavior adaptation in response to fear, *Tet1* governs alterations in 5hmC on synaptic plasticity genes during behavioral adaptation in response to stressful environmental exposures.

Taken together, these studies open up the possibility that 5hmC may function in the development of environmentally sensitive neuronal dysfunction. It will be of great interest to investigate the role of each *Tet* enzyme coupled with the rapid and stable dynamics of 5hmC at different developmental time points to understand its role in synaptic plasticity, neuronal development, the maintenance of mental health, and the onset of mental illness.

3.1 In the Origins of Psychiatric Disorders

Several studies indicate that early-life experiences have a profound impact on brain development and subsequent adult behavior (Oberlander et al. 2008; Mcgowan et al. 2009; Roth and Sweatt 2011b; Labonte et al. 2012). One such example involves rhesus macaques that were deprived of early-life maternal interactions. As adults, these monkeys have altered 5hmC in the prefrontal cortex on the promoter regions of genes related to neurological functions and psychological disorders (e.g., D_3 dopamine receptor (*DRD3*), serotonergic transporter (*5-HTT*), and GABAergic receptor (*GABRA2*)) (Massart et al. 2014). Since these 5hmC disruptions were detected

during adulthood, these findings suggest that early-life changes in 5hmC are stable throughout development and may represent the origins of developmental brain disorders such as SCZ, BD, and autism.

SCZ and BD are psychiatric disorders with shared and distinct clinical and genetic features; however, the majority of SCZ and BD cases *cannot* be explained by genetics alone. To investigate a role for 5hmC in SCZ and BD, genomic DNA and total RNA were obtained from the postmortem inferior parietal lobule (IPL) brain tissue of 10 SCZ patients, 9 BD patients, and 11 nonpsychiatric subjects, who had no history of psychiatric disorders. SCZ and BD patients exhibited increased 5hmC abundance and *TET1* expression, but not altered *TET2* or *TET3* expression (Dong et al. 2012). Remarkably, *TET1* was not altered in the cerebellum of these patients, suggesting that 5hmC may be involved in the development of psychosis through the inferior parietal lobule, but not the cerebellum, perhaps shedding light on the tissue-specific development of SCZ and BD. The increases of 5hmC in these patients were associated with reduced expression of biologically relevant genes including glutamic acid decarboxylase 67 (*GAD67*) and *APOBEC3A*, which plays a role in active DNA demethylation. Together, these findings suggest a common etiology in psychosis, one that includes genome-wide changes in 5hmC.

While associations between DNA methylation levels and MDD were described above, a recent study extracted genomic DNA from the inferior frontal gyrus of 19 clinically depressed suicide completers and 19 controls and measured genome-wide 5hmC levels. This approach revealed 550 differential hydroxymethylated sites in a plurality of genes, some of which also had differential expression, including myosin XVI (*MYO16*) and insulin-degrading enzyme (*IDE*), genes previously implicated in brain development and neurodegenerative disorders (Kurochkin and Goto 1994; Yui et al. 2015). These data shed light on an alternative molecular mechanism that may be involved in the development of MDD.

ASDs encompass a broad range of behaviorally related disorders with a high prevalence in children. Notably, only ~20% of ASD cases show a genetic etiology (Gaugler et al. 2014; Bulik-Sullivan et al. 2015). Prenatal factors shown to increase the risk of ASD in offspring include environmental influences such as multiple births, in vitro fertilization, and parental exposure to common drug treatments (e.g., antiepileptic drugs (e.g., valproate) or folic acid) (Gardener et al. 2009; Rogers 2008). Together, these findings effectively open the door for contributions from environmentally sensitive epigenetic modifications such as 5hmC to have an underlying role in the ASD etiology. Consistent with this hypothesis, genomic DNA extracted from one fetal and two human cerebellum brain tissues revealed that developmentally specific changes in 5hmC are highly enriched in known ASD genes (Wang et al. 2012b). In another study, the mRNA and 5hmC levels from the cerebellar cortex of ten ASD patients and ten controls were compared, and ASD patients had an enrichment of both *TET1* mRNA and 5hmC levels at the promoters of both *GAD67* and *RELN*, both candidate genes of ASD (Zhubi et al. 2014). More recently, genome-wide 5hmC levels were measured in cerebellum tissue from 17 ASD patients and 19 controls, revealing 797 age-dependent differentially hydroxymethylated regions (DhMRs) in the young group (age $\leq$ 18) and no

significant DhMRs in groups over 18 years of age, suggesting 5hmC may be affected across the spectrum early development and could contribute to the pathogenesis of ASD (Cheng et al. 2018). However, since all of these findings were observed post-symptomatically and in postmortem human brain tissue, it is unclear if the altered 5hmC represents a cause or a consequence of having autistic-like behaviors. Thus, these findings warrant a deeper investigation at pre-symptomatic developmental time points.

Finally, it is notable that rodent models exposed to prenatal stress exhibit long-lasting neurological, endocrinological, and behavioral changes that are thought to mirror the development of mental illness (Koenig et al. 2005). For example, prenatal stress increased anxiety-like behaviors and altered gene expression in mice heterozygous for the serotonin transporter (*5-HTT*) gene (Van Den Hove et al. 2011). Interestingly, these findings were more pronounced in female offspring, suggesting a sex-specific development of these altered behaviors. This study demonstrates a gene by environment interaction, and while this study did not examine 5hmC, the 5hmC-related data discussed in this review supports a 5hmC contribution to these altered behaviors and gene expression. If so, do these changes in 5hmC mirror those found in the homozygous mutants with advanced behavioral endophenotypes? This finding could shed light on the molecular mechanisms exacerbating early-life, stress-related behavioral outcomes in offspring that may have a genetic predisposition, a likely scenario for many mental disorders.

3.2 In Aging and Neurodegeneration

The emerging link between brain development and the accumulation of 5hmC with age has led researchers to examine this epigenetic mark in neurodegenerative diseases (Al-Mahdawi et al. 2014). Indeed, age-associated genes that acquire 5hmC are associated with pathways related to neurodegenerative diseases (Song et al. 2011). Human studies found that 5hmC was depleted in the hippocampus, cerebellum, and entorhinal cortex of ten patients suffering from AD, compared to ten controls (Chouliaras et al. 2013; Condliffe et al. 2014). On the other hand, studies of the middle frontal gyrus from 13 AD patients and the middle temporal gyrus from 29 AD patients revealed that an enrichment of 5hmC was positively correlated with hallmarks of AD, including neurofibrillary tangles (NFT), amyloid beta, and ubiquitin load (Coppieters et al. 2014). Together, these findings suggest that 5hmC may be driving the primary molecular components of AD progression within distinct regions of the brain. Notably, AD-associated levels of 5hmC also can be detected at preclinical stages of AD, as observed by the comparison of postmortem hippocampus and cerebellum brain tissues from five controls and five preclinical AD subjects, defined as having sufficient AD pathologic alterations at autopsy to meet intermediate or high NIA-RI criteria and moderate or frequent neuritic plaque scores according to the Consortium to Establish a Registry for AD (CERAD) with Braak scores of III–IV and antemortem psychometric test scores in the normal range when

corrected for age and education (Bradley-Whitman and Lovell 2013; Schmitt et al. 2000). These findings indicate that 5hmC may act as a viable biomarker of AD onset and progression. However, studies employing nuclear labeling or enzyme-linked immunosorbent assay (ELISA) were unable to find significant alterations in 5hmC associated with AD (Lashley et al. 2015), suggesting that higher-resolution methods are required to identify AD-associated changes in 5hmC. Connections between 5hmC and other neurodegenerative disorders including HD, ataxia-telangiectasia, and fragile X-associated tremor/ataxia syndrome have recently surfaced, and the link to 5hmC for each of these disorders is described below.

Recent epigenetic connections led to the investigation of the genome-wide distribution of 5hmC in a mouse model of HD, which found a deficiency of 5hmC in the striatum and cortex tissues (Wang et al. 2013). This altered distribution of 5hmC was generally associated with pathways involved in neuronal development/differentiation, axonal guidance, and neuronal function/survival. A specific example included a 5hmC reduction on the *ADORA2A* gene, a G-protein-coupled receptor that decreases its expression in HD patients ($N = 6$) compared to controls ($N = 5$), revealing that 5hmC may play a role in the pathological expression of *ADORA2A* found in HD patients (Villar-Menendez et al. 2013). Together, these results suggest that the loss of 5hmC explains the corresponding increase in total DNA methylation (5mC + 5hmC; see above) and provides a more complete understanding of a potential molecular mechanism underlying the cause of neuronal cell death associated with HD.

Ataxia-telangiectasia, a devastating neurodegenerative disorder that shows high cell specificity in its development, is caused by a mutation in the *ATM* gene (Mckinnon 2004). Despite this monogenic etiology, alterations in 5hmC were recently found in repeated sequences and regulatory elements in genomic DNA from *ATM*-deficient Purkinje cells of human patients ($N = 3$) compared to controls (Jiang et al. 2015). This 5hmC distribution is likely the result of TET1 responding to DNA damage, which is a hallmark of *ATM*-deficient neuronal cells. To test whether TET1 activity is related to Purkinje cell degeneration and behavioral deficits in mice, adenoviral particles encoding either human wild-type TET1 (TET1-WT) or kinase dead mutant TET1 (TET-KD) were infected into *ATM*-deficient mouse cerebellum slices and controls. Cerebellum samples lacking TET1 expression that were infected with TET1-WT showed no activation of *Caspase-3*, a cell death marker, while infection with TET1-KD activated *Caspase-3*. These findings suggest that TET1-mediated 5hmC production is essential for Purkinje cell viability and the prevention of ataxia-telangiectasia-like symptoms in mice, supporting the concept that vulnerability to neurodegeneration is linked to aberrant changes of 5hmC in neuronal cells.

Fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder, is one of the characterized fragile X disorders that are caused by a CGG expansion in the 5′UTR of the *FMR1* gene (Hagerman and Hagerman 2015; Santoro et al. 2012). Genome-wide analysis of 5hmC in the cerebellum of a FXTAS mouse model (rCGG mice) revealed an overall reduction in 5hmC at 16 weeks of age when compared to age-matched controls (Yao et al. 2014). Despite the overall reduction of 5hmC, these mice have an increase of 5hmC in repetitive sequences

as well as in cerebellum-specific enhancers. Differential 5hmC between the rCGG and control mice were predominantly found in transcription factor (TF) binding sites that are located in genes essential for neuronal development. Finally, ribosomal profiling revealed that the differential 5hmC-associated genes often exhibited altered ribosomal processing in the rCGG mice, suggesting that 5hmC may somehow influence translational changes. In summary, this study links 5hmC to the etiology of FXTAS and implicates a role for 5hmC in transcription factor binding and in regulating ribosomal processing of mature RNA transcripts.

Despite that the above disease-associated disruptions in 5hmC represent changes across age, these examples clearly demonstrate a role for 5hmC in development and in the onset and progression of neurodegenerative diseases. These developmental and neurodegenerative disease-associated changes in 5hmC often arise within distinct cell types and brain regions, supporting cell- and tissue-specific development of these diseases. Since these studies have been largely descriptive, it is imperative that future studies determine the functional mechanism(s) played by 5hmC if we are to modify it toward healthy outcomes.

4 Clinical Utilities of DNA Methylation

Diagnosis and risk assessment of psychiatric disorders are typically performed using family history, subjective symptom reports, and behavioral observations, which are vulnerable to the variability in accurate patient reporting and consistent clinician interpretations. Thus, molecular biomarkers are being sought because they represent objective measures that can more accurately diagnose and stratify behavior disorders. For example, suicide biomarkers could accurately distinguish between a pathological mood disorder and a normal symptomatic response to an acute stress (e.g., bereavement). These biomarkers also may provide insight into the underlying molecular mechanisms contributing to specific mood-related behaviors. As another example, a recent study examined the peripheral blood from PTSD patients with matched adult trauma exposure and found distinct PTSD-associated DNA methylation profiles, which were related to their different childhood adverse events ($N = 32$ and 29) (Mehta et al. 2013). These findings suggested that DNA methylation levels across the genome might reflect differences in the pathophysiology of PTSD. Ultimately, this information can provide much needed insight into treatment response and the development of novel therapeutics aimed at alleviating mood disorders.

DNA methylation provides a highly sensitive and dynamic biomarker in real time, immediately marking exposures to various environmental stimuli such as stress, cognitive behavioral therapy (CBT), electroconvulsive therapy (ECT), antidepressant treatment, and exercise. Moreover, it is notable that environmentally sensitive DNA methylation levels can be stable across generations. Thus, the methylome is a promising candidate to be an objective biomarker of human behavior following environmental stimuli. Recent support for using DNA methylation as an

objective biomarker of environmental stimuli came from a study that examined the genomic DNA from two cohorts of urban African Americans, from the Grady Trauma Project ($N = 421$) and the Johns Hopkins Center for Prevention Research Study ($N = 326$), and found an interaction between blood *SKA2* methylation and trauma scores (particularly childhood emotional abuse). This interaction was successful at predicting suicidal ideation and suicidal behavior with 71% and 73% accuracy, respectively (Clive et al. 2016). Notably, prediction of suicidal ideation and behavior from saliva samples also was promising, as *SKA2* methylation levels following childhood abuse had a 76% accuracy in predicting suicidal ideation. On the other hand, saliva *SKA2* methylation is linked to extreme anxiety and had 69% accuracy in predicting suicidal ideation, further supporting the sensitivity of DNA methylation to distinguish between experience-induced symptomatic responses. In the same study, the *SKA2* methylation prediction model was used to predict PTSD in the Grady Trauma Project cohorts. Interestingly, while *SKA2* methylation had 72% accuracy in predicting PTSD that was defined by the Child Trauma Questionnaire (CTQ) scores, *SKA2* methylation alone only had 55% accuracy in predicting PTSD. Together, these data suggested that combining DNA methylation information with rigorous behavioral assessments improves our power to diagnose mood disorders. Other researchers sought to further improve this suicide prediction model by determining if DNA methylation levels at other genes correlate with suicide. These studies revealed that the DNA methylation levels of three genes (*DDR1*, *ARHGEF10*, *SHP1*) correlated with suicide and *SKA2* methylation levels and together they could consistently predict suicide across all tested data sets, including youth at high risk for depression, pregnant women at risk for elevated postpartum depression, and middle-aged individuals with a high incidence trauma and PTSD. Together, these findings shed light on the possibility for standardized screening with a single objective test, perhaps without the need for a subjective behavioral assessment.

Another promising example of using DNA methylation to diagnose and stratify risk for behaviors includes recent work that investigated fear extinction in patients suffering from panic disorder. This study examined genomic DNA from blood of female patients that received CBT for 6 weeks ($N = 28$), panic disorder patients that did not receive CBT ($N = 20$), and controls ($N = 28$) and found that the DNA methylation on the monoamine oxidase A (*MAOA*) gene was inversely associated with panic disorder severity (as measured by either panic attacks or severity of agoraphobia symptoms). Perhaps most exciting, *MAOA* methylation levels responded to CBT, as the *MAOA* methylation in patients undergoing CBT matched the levels of controls. These findings indicate that *MAOA* methylation levels may be physiologically linked to the therapeutic effects of CBT, with the simplest interpretation being that increased *MAOA* methylation results in decreased *MAOA* gene expression and increased bioavailability of monoamines in the synaptic cleft. Clinically, this could allow for an objective screening tool for panic disorder symptoms and for monitoring the efficacy of panic disorder treatment.

The sensitivity of 5hmC levels following prenatal and/or acute stress underscores the potential for 5hmC as a novel biomarker in the diagnosis of mental health. In

addition, the presence/absence of these epigenetic modifications is reversible; thus, they may become relevant in therapeutic interventions (Szyf 2015), especially if methods to selectively modulate 5hmC in vivo are developed at the nucleotide level. Finally, 5hmC has sex-specific profiles, which is of interest because the development of several psychiatric disorders is seemingly sex-specific. For example, females show an increased risk in developing anxiety and depression, while males show a disposition to development of attention deficit hyperactivity disorder (Nolen-Hoeksema 1987; Wooten et al. 2004). A recent study found sex-specific 5hmC on genes with ontological terms correlating with organ morphogenesis, system development, and development of anatomical structures (Gross et al. 2015), suggesting that 5hmC may differentially influence the development of organs (e.g., the brain) in the sexes. Together, these factors must be considered for clinical application and treatment endeavors.

5 Conclusions

The findings described in this chapter highlight the research that has been conducted toward improving our understanding of the molecular events contributing to the development of mental health and illness. Importantly, these studies promote scientific questions that are less limited by the bounds of environment versus genetic, or categories of biologic versus psychiatric, and may spawn productive directions toward improved standard of care in psychiatry. Perhaps most promising is the quantitative nature of 5mC and 5hmC detection, indicating that these marks show promise to serve as unifying links between tissue-level activity and complex behavior. Surprisingly, the methylome is highly sensitive to environmental stimuli, showing alterations in as little as 30 min. Moreover, these marks are both stable and reversible – as shown in suicide completers and in cocaine addiction model, respectively. Harnessing the methods to control this reversibility is of great interest, and some examples were presented, which included decreases in methylation levels in patients with BPD following dialectic behavioral therapy or rodents in response to arched back nursing or enhanced maternal care. Fortunately, brain imaging methodologies such as BOLD and fMRI have confirmed that methylation levels in the blood are linked to tissue activity in the brain. This finding has enabled other studies to test a variety of tissues from which samples can be examined – expanding from brain tissue to blood and buccal swabs – allowing for more flexibility including the ability to obtain epigenetic data from living human subjects. While we have yet to fully understand the functional roles of DNA methylation and hydroxymethylation in behavior, we are beginning to recognize that environmentally sensitive alterations in the methylome are linked to gene expression changes governing behavior. The need for such understanding is especially clear in conditions where pathogenesis is not explained by genetics alone: as in autism, which is thought to be only ~25% explained by genetics; SCZ, MDD, and PTSD, for which monozygous twins can be discordant; and suicide, where predisposition is known to have a significant

interaction with environmental factors, such as life experiences and history of trauma. We hope that further understanding and study of the methylome will allow us to improve classification, detection, treatment, and prevention of psychiatric conditions.

Disease classification is an area of constant clinical and research interest. The *Diagnostic and Statistical Manual of Mental Disorders* (DSM-V) guides diagnosis of psychiatric conditions, almost exclusively using behavioral symptoms and signs. This manual, while carefully crafted and painstakingly edited longitudinally by experts in the field of psychiatry, remains vulnerable to subjectivity. It is well documented that many conditions can confound the clinical picture of others; for example, anxiety can share symptoms with ADHD, yet the treatment of one can exacerbate the other. In the future, we hope investigators seek objective biomarkers to aid in classification of mental health and believe that the methylome offers a potential means toward the goal of providing a more personalized diagnosis that will lead to more precise/effective treatments (Fig. 4). This scenario will eliminate the diagnostic odyssey that many mental illness patients endure and provide a rapid treatment strategy toward a healthy outcome.

We also anticipate that the early detection of mental illness will be an important clinical application of the methylome. An example where a molecular indicator, like the methylome, would be effective is in the "prodrome" phase of a disorder (e.g., SCZ), when a patient presents with a subtle abnormal behavior that does not indicate a clear diagnosis. In addition, early detection leads to early treatment, which can dramatically improve outcomes for most mental illness patients.

The current standard of care for the detection of a mental illness is limited, relying heavily on patient reporting and symptom recognition. Among future areas for research, we see an opportunity to identify novel quantitative indicators for risk, through identifying aberrations to the methylome which can be measured peripherally in a clinical setting, perhaps through leukocytes via blood draw or buccal swab. We already have observed a relationship between the methylation levels in the promoter region of the oxytocin receptor gene and autism and *BDNF* in suicide completers. However, much work remains to tease out correlations between genes in multiple disorders, such as why increased DNA methylation levels on the *OXTR* promoter region is associated with autism yet decreased DNA methylation levels on the *OXTR* promoter region is associated with social anxiety disorder – much like copy number variations? Can DNA methylation levels lead to different disorders based on too much or too little? We anticipate that improved understanding of the complete methylome may lead to clinically useful biomarkers for risk of a variety of psychiatric illnesses that span the lifetime. This knowledge of the methylome and its role in etiology of psychiatric conditions may allow us to identify unique physiologic targets for pharmacological intervention. We can likewise develop more targeted nonpharmacologic therapies and apply them with more specificity. In short, the methylome represents an exciting frontier that can lead to more effective collaboration between the individually developed fields of psychology, psychiatry, neuroscience, and genetics. This unification of understanding may potentially lead to more objective and precise disease classification, risk stratification, treatment, and ultimately improved quality of life for vulnerable individuals and populations.

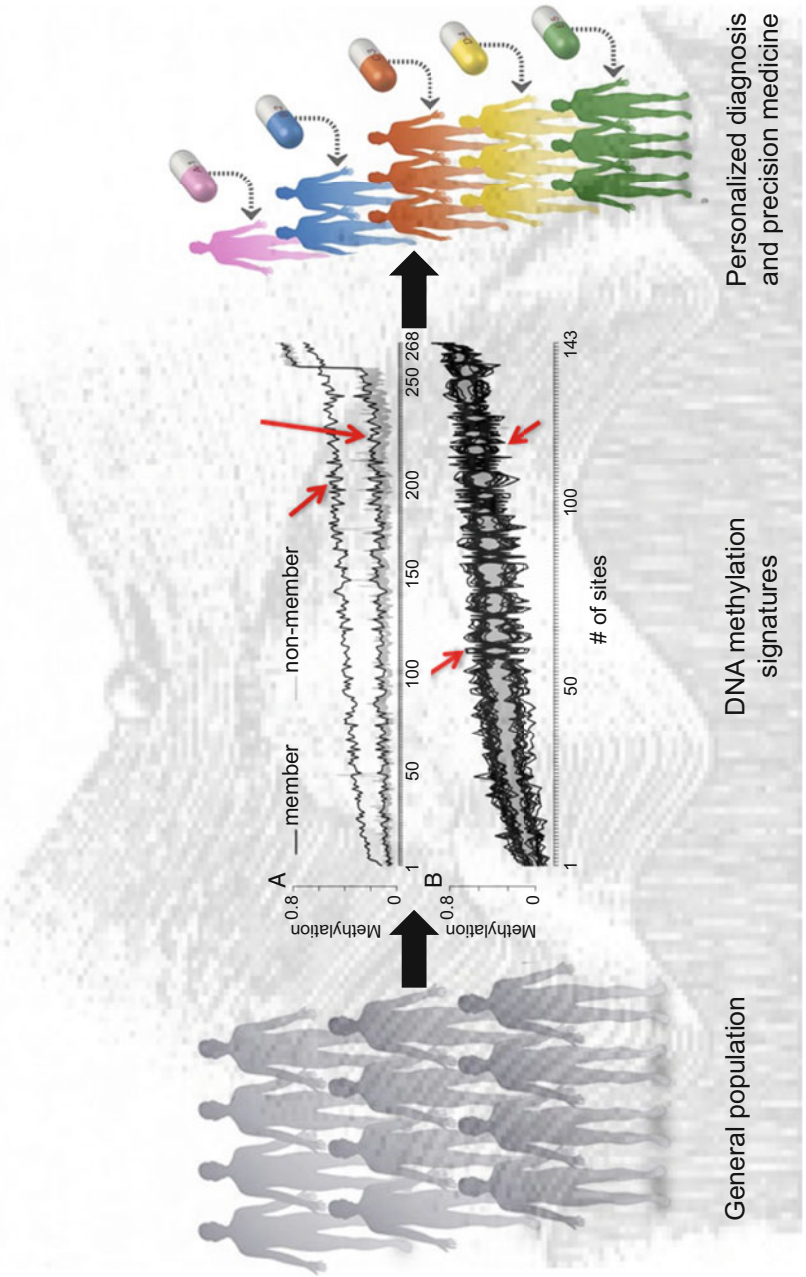

Fig. 4 Improving the standard of care in psychiatry. Genome-wide signatures of DNA methylation can be used to stratify the general population. This process can result in a personalized diagnosis that is more rapid and exact. Moreover, it will provide insight into the genes and pathways contributing to the individual diagnosis, which will guide more precise treatment for the individual

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Social Environment and Epigenetics

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Contents

Abstract Our social environment, from the microscopic to the macro-social, affects us for the entirety of our lives. One integral line of research to examine how interpersonal and societal environments can get "under the skin" is through the lens of epigenetics. Epigenetic mechanisms are adaptations made to our genome in response to our environment which include tags placed on and removed from the DNA itself to how our DNA is packaged, affecting how our genes are read, transcribed, and interact. These tags are affected by social environments and can persist over time; this may aid us in responding to experiences and exposures, both the enriched and the disadvantageous. From memory formation to immune function,

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the experience-dependent plasticity of epigenetic modifications to micro- and macro-social environments may contribute to the process of learning from comfort, pain, and stress to better survive in whatever circumstances life has in store.

Keywords Adversity · Built environment · DNA methylation · Enriched environment · Epigenetics · Histone modifications · Interpersonal · Learning · Pain · Parenting · Social environment · Stress

1 Introduction

To be human is to have a fundamental need for love and belonging. Humans give birth to altricial young who depend on the effectiveness and attentiveness of social bonds from the moment of delivery, not only to thrive, but also to survive. Though this is a unique trait among mammals, it also means humans are exposed to their social environment much earlier in development than most offspring in the animal kingdom. Children's social relationships are arguably the most fundamental component of the early postnatal environment and facilitate both the beneficial and harmful effects of ecological factors. Even meeting necessary nutritional needs through breastfeeding is a social bonding experience with close proximity, ventro-ventral contact, body warmth, and soft touch. As the preeminent developmental psychologist Urie Bronfenbrenner stated: "No society can long sustain itself unless its members have learned the sensitivities, motivations, and skills involved in assisting and caring for other human beings" (Bronfenbrenner 2009).

All children develop within a dynamic social context of both interpersonal relationships and wider social structures, which can shape their cognitive, emotional, and biological processes for the remainder of their lives (Casper 2001). From the first moments with parents to city planning to feeling accepted by the community, the multifaceted nature of the social environment provides ample opportunity for both advantages and hindrances to be embedded "under the skin" (Boyce and Kobor 2015).

Though the debate between solitary contributions of nature and nurture is an academic artifact, there are a variety of overlapping conceptualizations of this embedding process. For example, the concept of vulnerability and resilience, or resistance, is commonly used in psychology and psychiatry (Ingram and Luxton 2005; Luthar 2003), as well as in engineering and ecology (Miller et al. 2010; Proag 2014). These terms have similar meanings even in these disparate fields with vulnerability referring to the underlying potential of an adverse reaction to a negative event, and resilience referring to the innate ability to withstand and recover from a negative event. This is the same principle as an individual genetic predisposition for a particular outcome, either beneficial or detrimental. Similarly, the psychological diathesis-stress model, or dual-risk model, presumes a predisposition that affects the likelihood of developing, or the trajectory of, a disorder (Ingram and Luxton 2005). This diathesis may be genetic, biological, environmental, or psychological and

interacts with some form of biological or environmental stress to alleviate or exacerbate the effect. This also resembles the threshold model of neurobiological reactivity (Moore and Depue 2016). This model proposes that there is a biological constraint on the potency of external stimuli necessary to elicit an emotional response. This model presupposes that there is a predisposition to be stressed by an environment in the first place, in addition to the possibility that the subsequently elicited stress response may exacerbate yet another outcome. This cascade of interactions between innate differences and environmental exposures is addressed in the developmental origins of health and disease (DoHaD) hypothesis (Mandy and Nyirenda 2018; Suzuki 2018). This hypothesis refers to the potential biological programming from environmental exposures that may cause some of the intrinsic predispositions on which later environments may act (Wadhwa et al. 2009).

The most general and often used term for these relationships is gene-by-environment interactions. Ultimately, the distillation of these models equates to a basic understanding that the scaffolding of experience can be secured upon existing biological foundations. With poor foundations or inferior craftsmanship, the overall integrity of the structure may fail. One important aspect of how these pieces come together to build the human experience is through epigenetics (Meaney 2010).

2 Epigenetic Modifications

Epigenetics refers to the varied modifications to the underlying, permanent deoxyribose nucleic acid (DNA) sequence, which subsequently alter gene expression and, ultimately, phenotypes such as health and behaviors. The fundamental purpose of these epigenetic alterations is to achieve a diverse landscape of expression from a single DNA source (Boyce and Kobor 2015). These changes are likely involved in the biological embedding of environmental influence because of both their dynamic nature and sensitivity to experiential feedback. The field of epigenetics is a natural accretion of biological reductionism as it provides evidence that while we may be a product of our biology, our biology is partially a product of our environment.

The most common forms of epigenetic investigations in humans are correlational studies on DNA methylation (mC) and DNA hydroxymethylation (hmC), as discussed elsewhere in this volume. Due to its critical role in cell type differentiation, mC is highly affected by cell type differences and, thus, tissue types, as well as age, ethnicity, genotype, sex, and disease state (Edgar et al. 2017; Hannon et al. 2015; Husquin et al. 2018; Islam et al. 2019; Lienert et al. 2011; Turinsky et al. 2019; Wagner et al. 2015; Yousefi et al. 2015; Ziller et al. 2013). Three of these factors are especially important to highlight: genotype, tissue, and age. When considering genotype, de novo modifications may be affected by the underlying DNA sequence due to potential sites of interaction, a downstream consequence of other modifications, or the result of adaptation to environmental influences that can be passed on through cell mitosis to have long-lasting effects (Bock et al. 2006; Probst et al. 2009; Song et al. 2017). When considering tissue, the two most important aspects are

differences in cell type and cell type proportions among the different tissues and the comparisons that can be made among and between different tissue types. Specifically, it is difficult to make inferences about epigenetic modifications in brain tissue when measuring more peripheral tissues such as blood or saliva; however, there are resources correlating some measures in these tissues that can assist in forming educated inferences (Braun et al. 2019; Edgar et al. 2017).

Finally, when considering age, both its potential as a confounder and variable of interest should be acknowledged. Patterns of epigenetic modifications change with age, including mC (Gopalan et al. 2017; Jones et al. 2015; Koch and Wanger 2011; McEwen et al. 2016, 2017; Sen et al. 2016). Therefore, it is crucial that age be accounted for, selected for, or counterbalanced across groups in the variable of interest. A subsequent benefit of these clear and replicable differences in ages, however, is the ability to predict age using DNA methylation "clocks" that can be both tissue specific and pan-tissue (Horvath et al. 2016; Horvath and Raj 2018; Liu et al. 2019; Marioni et al. 2015; Wagner 2017). By calculating age using DNA methylation, it is also possible to determine the acceleration or deceleration of a person's epigenetic age from their chronological age. Generally, when an adult is predicted to be older than their chronological age (i.e., epigenetic age acceleration), this suggests increased cellular aging and is associated with increases in morbidity and mortality (Fransquet et al. 2019; Horvath et al. 2015; McEwen et al. 2016).

While the field is acutely aware of the importance of accounting for these potential confounders, many early foundational studies in social epigenetics did not account for these differences and are primarily correlational, observational designs. Additionally, due to the difficulty, cost, and technological limitations in conducting epigenome-wide association studies (EWAS), much of the initial work focused on a candidate gene approach. The previous literature on mC in candidate genes should not be disregarded; however, new appreciation of the interconnectedness among mC indicates the benefit of EWAS due to accounting for differences among many correlated sites or regions simultaneously (Moore 2017). While there is crucial work to be done in understanding how social environments affect the underlying biology of developmental sequelae, a healthy dose of skepticism and a critical eye must be maintained both when evaluating past and current literature, as well as developing new experimental designs (Jones et al. 2018). There are also other possible DNA modifications that can affect gene expression that have been significantly less studied than mC. Although not much is known about their relationships with early social environments, additional cytosine modifications formylation and carboxylation, as well as the much rarer methylation at adenine sites on DNA, are ripe for future investigation (Wu et al. 2016b; Yao et al. 2017). Besides modifications directly onto DNA base pairs, there are other ways to affect the complex relationship between DNA structure and protein synthesis.

One such way is to affect the packing of DNA through modifications to chromatin, the condensed DNA-protein package that allows a structure as large as DNA to reside within the nucleus of a cell. Within the chromatin package are an octamer of proteins called histones. This package contains two each of four types of histones: H2A, H2B, H3, and H4, the tails of which have at least 14 possible modifications

known to date (Bártová et al. 2008; Huang et al. 2014; Kouzarides 2007). By modifying chromatin structure, the accessibility of the DNA for gene transcription is significantly affected. This is a metric referred to as chromatin accessibility (Buenrostro et al. 2015, 2016; John et al. 2011). One modification that usually increases chromatin accessibility, thus increasing the ability of transcription factors to bind to the DNA, is histone acetylation (Görisch et al. 2005). This is when acetyl groups are deposited on lysines by histone acetyltransferaces (HAT). Alternatively, these groups that typically act to release compacted DNA to facilitate transcription initiation can be removed by histone deacetylases (HDAC), thus reducing chromatin accessibility (Chen and Townes 2000; Shahbazian and Grunstein 2007). There is significant evidence of histone acetylation and deacetylation having social behavioral effects in animal studies (Bukhari et al. 2017; Fitzsimons and Scott 2011; Hunter et al. 2012; Malik et al. 2014; Peixoto and Abel 2012; Saul et al. 2017; Shpigler et al. 2017).

Another possible histone modification is phosphorylation, which is often associated with acetylation, and functions primarily in the histone microenvironment by serving as a platform for communication between other histone modifications and downstream effects (Banerjee and Chakravarti 2011). Phosphate groups are customarily deposited by nuclear kinases and removed by protein phosphstases (Brami-Cherrier et al. 2009; Koshibu et al. 2009). Similar to DNA, histones can also be modified to be methylated, which, like DNA, can lead to both increased and decreased chromatin accessibility depending on the modified site (Kouzarides 2007). Methyl groups are deposited on histones by histone methyltransferases (HMT) and removed by histone demethylases (HDM) (Bártová et al. 2008; Zheng et al. 2015). In addition to their interactions with one another, these modifications are affected by the principle DNA structure, histone chaperones, age, and histone protein variants such as the H2A histone variant H2A.Z (Ausió and Abbott 2002; Bryois et al. 2018; Levine et al. 2012; Mcvicker et al. 2013; Stefanelli et al. 2018; Tessarz and Kouzarides 2014).

Once the DNA has been made available to transcription factors through variations of, and modifications to, DNA, chromatin, and histones, there is yet another epigenetic mechanism which takes place on ribonucleic acid (RNA). Messenger RNA (mRNA) acting as an intermediary between the underlying DNA structure and the protein synthesizing ribosomes, just like histones and DNA, can be modified by the addition or subtraction of hydroxymethyl, acetyl, phosphate, and the most frequently studied, methyl adenine (m^6A) groups (Boccaletto et al. 2017; Meyer and Jaffrey 2014; Saletore et al. 2012). These modifications, sometimes referred to as epitranscriptomics, can affect the structure, binding, transcription, and translational properties of RNA, which in turn has significant implications for downstream protein synthesis (Schwartz 2016). There are also two other families of RNAs that can have significant effects on gene transcription without affecting the underlying DNA sequence: microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). Both miRNAs, which act as complimentary sequences to degrade mRNA and lncRNA, which regulate mRNA, generally reduce transcription and are affected by the underlying DNA sequence (Chu et al. 2011; Kim 2005; Kim and Nam 2006; Lee

2012; Mercer et al. 2009; Younger and Corey 2011). The direct relationship of the social environment on miRNAs and lncRNAs is currently unknown.

This diverse wealth of epigenetic mechanisms does not operate in a vacuum but functions together to have a cascade of comprehensive and varied influences. These modifications can be preserved for a lifetime through biological machinery that accurately maintains the epigenetic pattern (Probst et al. 2009), thus providing the opportunity for dynamic early environmental epigenetic adaption to be maintained throughout a lifetime. Therefore, there are many known epigenetic mechanisms that would allow for the possibility of early life social environments to stably modify phenotypes over the life course without altering the genotype. In this chapter, we discuss the scope of varied epigenetic investigations into the biological consequences of both interpersonal and structural social environments. To begin, we focus on the intersection of social behavioral neurogenomics: learning.

3 Epigenetics and Learning

The social environment is, fundamentally, an inescapable impetus for development and adaption through learning and memory, both positive and negative. Infants are innately attuned to social cues, even before birth. Infants have a strong preference for eyes, faces, and facial configurations (Dupierrix et al. 2014; Goren et al. 1975; Johnson et al. 1991, 2015; Turati et al. 2002). Even third trimester human fetuses preferentially orient toward a face shape when projected through maternal tissue (Reid et al. 2017). This strong preference can be interpreted as an indication of the importance of early exposure to faces and an early orientation toward social stimuli (Morton and Johnson 1991). In addition to the infant's predilection for faces, young infants develop in an especially face-dense environment, even in comparison to older infants (Jayaraman et al. 2017). As young infants have a low range of mobility, this demonstrates an implicit adult drive to place faces in front of developing infants as well. Infants also use touch to communicate with their caregivers and use a variety of movements depending on their needs and the caregiver's responsiveness (Moszkowski and Stack 2007). Additionally, evidence indicates maternal odors enhance neural signaling of facial categorization, but not general activation, in infant brains (Leleu et al. 2019).

Babies' relationship with the social environment is a multi-sensory process. In addition to sight, smell, and touch, infant's hearing is also highly attuned toward social environments and language development. Adults tend to use infant-directed speech (IDS) when with children, which incorporates a larger range of higher frequencies and has more rising contours than adult-directed speech (ADS). Infants are more attentive to, can discriminate sounds better in, and can be emotionally regulated by IDS as opposed to adult-directed speech (Cooper and Aslin 1990, 1994; Fernald 1992; Trehub et al. 1993). Even mothers of deaf children use exaggerated signs to their infants, who are more attentive to the exaggerated infant-directed

signing (Masataka 1998). An infant's world is full of social stimuli to which they can experience, react, and learn.

This rich social environment is used by infants to both survive, via their caregivers, and begin to build their understanding of the world. Therefore, it logically follows that children's cognitive functional development, both normative and deviant, adapts to the social environment (Dishion 2016). Children show a novelty preference almost immediately and are able to respond to traditional behavioral conditioning paradigms (Hulsebus 1974; Thompson et al. 1991). Infants as young as 6 months can reliably show social learning through deferred imitation (Barr et al. 1996; Meltzoff 1988). Additionally, experiential learning generally is essential for normative development both pre- and postnatally (Bale et al. 2010; Mclaughlin et al. 2014; Perry 2002; Roth and Sweatt 2011a, b; Swain et al. 2007; Talge et al. 2007). Learning from and adapting to the early life environment as effectively as possible is crucial for both juvenile and adult survival. It is both demonstrable and logical that children would be learning from their environments, and it is clear that there is an emphasis on the social environment for those associations.

Some connections, such as those learned or imitated in an interpersonal social context, are more readily learned than others. This is called biological preparedness theory, and the belief is that there is an evolutionary advantage to more quickly making these environmentally and survival-relevant associations (Cummins and Cummins 2015; McNally 2016). It is also much more difficult to extinguish these connections once learned (Åhs et al. 2018). Most work has focused on preparedness role in phobias, anxiety disorders (including social anxiety), and taste aversion (de Silva 1988; Ohman and Mineka 2001). Due to the importance of social bonds to human survival, especially for young children, and the extreme innate infant preference for social stimuli, the assumption of an underlying biological preparedness to learn cues from the social environment is reasonable. Albert Bandura, a distinguished behaviorist, acknowledged the unique aspects of social environments. He adapted the behavioral conditioning theory to include the observational process of learning and noted that there are mediating processes between the experience of a stimuli and subsequent response (Bandura 1977). The cognitive ability of children to learn from, and disproportionate attunement toward, their early social environments indicate that these social factors are primed to make lasting neurological, epigenetic, and behavioral impressions.

In order to leave these lasting impressions, first the associations from the social environment need to be learned. The basis for most models of development, learning, and memory in neuroscience is synaptic plasticity, or the ability of synaptic connections to change their strength (Ehrlich and Malinow 2004). Hebbian theory, or cell assembly theory, posits that two mechanisms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) (Ehrlich and Malinow 2004; Robert C Malenka and Nicoll 1999; Randic et al. 1993). Electrophysiological action potentials are primarily responsible for, or supportive of, cell-to-cell communication through the synapse in the nervous system through rapid cell depolarization that is passed along to adjacent cells.

LTP is one cellular mechanism through which the strength of a synapse can be amplified, while LTD is a mechanism for weakening a synapse. LTP primarily

occurs through the action of presynaptic glutamate at two types of postsynaptic ionotropic glutamatergic receptors, N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Malenka and Nicoll 1999). Though glutamate binds to both NMDA and AMPA, NMDA receptors are gated by a magnesium ion block that prevents calcium ions, to which NMDA receptors are permeable, from entering the postsynaptic neuron. However, AMPA receptors do not have a magnesium block and are activated by presynaptic glutamate release, allowing sodium ions to flow through. When enough AMPA receptors have been activated and sodium ions have entered the postsynaptic neuron, the charge, or potential, of the neuron changes, releasing the magnesium ion block from the NMDA receptors. The NMDA receptors then allow calcium ions into the cell, which target calcium/calmodulin-regulated protein kinase II (CaMKII) (Malenka et al. 1989). CaMKII phosphorylates AMPA receptors, increasing their current, a mechanism of early LTP, and initiates protein synthesis through the MAPK (mitogen-activated protein kinase) pathway and CREB/CRE (cAMP-responsive element binding protein/cAMP response element) mechanisms to begin structural changes in and around the synapse (Giese et al. 1998).

Early LTP results in the addition of more AMPA receptors postsynaptically and a retrograde signal of nitric oxide to the presynaptic neuron, while late LTP adds an entirely new synapse between the two neurons (Sandkühler and Gruber-Schoffnegger 2012). BDNF (Brain-derived neurotrophic factor) is crucial in this process, with reduction leading to insufficient drive for synthesis of synaptic proteins, thus contributing to cognitive dysfunction (Wu et al. 2016a). Both adding more AMPA receptors, thus depolarizing and triggering NMDA receptor activation in the postsynaptic neuron, or adding a new synaptic cleft, increases the strength of the connection between the two neurons. This strengthened connection is sensitized to be activated again and the presynaptic cell is poised to interact with the postsynaptic neuron more quickly and strongly than before.

LTD, on the other hand, takes place when there is consistent low frequency activation. With constant low activation, only some, but not all, AMPA receptors are activated, which is not enough to remove the magnesium ion block from the majority of NMDA receptors. In this case, calcineurin (protein phosphatase 2B) is the target, which results in the removal of AMPA receptors by endocytosis (Malenka and Bear 2004). Though LTD is the weakening of synaptic strength, it plays an integral role in memory formation, most likely through preparing potential pathways for new connections. Additionally, spike-timing dependent potentiation (STDP) adds even more specificity to this relationship by altering the strength of LTP and LTD effects depending on the timing of electrical signals (Fiete et al. 2010). The strengthened relationships among neurons happen within milliseconds and can last from 30 min to years. LTP in multiple synapses can create engrams, or biophysical manifestations of memories, stored as cognitive units of interconnected cells (Poo et al. 2016). The maintenance of these engram connections composes long-term memory. How are these changes generated in the first place? How are they maintained over multiple cell turnovers and conceivably for an entire lifetime? One possibility is through epigenetic mechanisms that alter gene transcription to allow for stable, structural modifications to the synapse.

Contributing to the neuroarchitectural changes associated with learning, brief and distinct changes to neural gene expression are observed, termed genomic action potentials (gAP) (Clayton 2000; Clayton et al. 2019). The pathways activated by calcium interacting with CaMKII and calcineurin lead to increased gene transcription of elements necessary for cytoskeletal changes, termed the immediate early gene (IEG) response (Clayton et al. 2019). This dynamic change results in epigenetic modifications at the transcriptional and chromatin packaging levels (see Clayton et al. 2019 for review). One example is *ARC*, an IEG, which is involved in the endocytosis of AMPA receptors seen following calcineurin activation in LTD (Chowdhury et al. 2006; Rial Verde et al. 2006). Another example of IEG action is *FOS*, which interacts with histone methylation to prime histone acetylation based on neuronal activity. These common IEGs are poised to have quick transcriptional changes in multiple domains such as histone lysine modifications and mRNA interactions (D'Urso and Brickner 2014; D'Urso et al. 2016; Rye et al. 2014). It is also reasonable to hypothesize that, as there are differences in synaptic plasticity depending on the types and locations of neurons, as well as temporal differences such as early vs. late LTP or STDP, this is also true for the IEG associated with these changes (Tyssowski et al. 2018). Additionally, like the pattern of synaptic connections, the gAP differs when encoding different learning experiences (Mukherjee et al. 2018).

Histone and mC modifications, in particular, have been associated with synaptic plasticity and learning outcomes (Blaze and Roth 2013; Chwang et al. 2006; D'Urso and Brickner 2014; Dias et al. 2015; Feng et al. 2010; Kim and Kaang 2017; Levenson and Sweatt 2011; Peixoto and Abel 2012) as well as affording significant contribution to differences in the neural architecture of neonatal socioemotional learning (Ong et al. 2019). Consistently, pharmacological inhibition of mC modifications significantly impairs memory, and many EWAS identify mC modifications in genes involved in neurotransmitter pathways and learning (Day and Sweatt 2011; Mill et al. 2008).

Additionally, increases in histone acetylation and alterations in histone methylation are associated with memory formation, synaptic plasticity enhancement, and increased gene expression (Guan et al. 2002; Pang et al. 2019; Schaefer et al. 2009). For example, oral administration of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) restored spatial memory and reduced inflammation in an aging animal model (Benito et al. 2015). There is also evidence that histone lysine methyltransferase complex G9a/GLP facilitates LTD maintenance in the hippocampus and inhibiting HATs significantly impairs learning potential (Oliveira et al. 2007; Sharma and Sajikumar 2018). A specific example of the role of histone methylation is the reduction of H3 histone dimethyl occupancy in the promoter region of a gene associated with long-term memory formation, *HOMER1A*, and an increase in its transcription in the amygdala during auditory fear conditioning (Mahan et al. 2012). Conversely, an increase in HDAC activity results in reduced synaptic plasticity and memory impairment, while pharmacological inhibition of HDAC improves memory formation (Guan et al. 2009; Levenson et al. 2004). Also,

histone acetylation in the hippocampus is a consequence of contextual fear conditioning in rodent models (Levenson et al. 2004).

Another aspect of biological memory beyond encoding and maintenance of explicit and implicit learning is homeostatic conditioning (Clayton et al. 2019). Homeostasis is a summary mechanism maintaining a steady biological equilibrium to best preserve an optimal internal environment (Martin 2008). Depending on the changes to internal and external states, it is sometimes necessary to rebalance these homeostatic systems to reach a new equilibrium. In this case, a synapse changes because of homeostatic mechanisms. A common and illustrative neuronal example of homeostatic compensation is gaining tolerance to opiates (Koob 1996). By activating mu-opiate receptors in the brain at a constant, elevated level, these receptors become internalized and, eventually, broken down, while receptors responsible for opposite reactions, like norepinephrine receptors, are upregulated through increased transcription (Finn and Whistler 2001). This new receptor balance is a reaction to the ingestion of exogenous opiates and is responsible for both increasing tolerance to and withdrawal symptoms from opiates. Similar to Hebbian learning, these fluctuations could not occur without dynamic and maintained changes in gene transcription. Therefore, Clayton et al. (2019) posit an additional function of the gAP which could be regulating homeostatic compensation in a similar fashion. Homeostatic mechanisms working through the gAP at the network level may be more likely in response to large variations that activate homeostatic receptors such as changes in developmental stage, drug ingestion, or significant environmental experiences (Hrvatin et al. 2018; Miyashita et al. 2009; Miyatake et al. 2005; Tyssowski et al. 2018).

One example in humans of genetic deficits in epigenetic modifications resulting in profound cognitive and social dysfunction is Rett Syndrome (Jiang et al. 2004). Rett Syndrome is an intellectual disability disorder that results from a mutation in the *MeCP2* gene, which checks and binds areas of DNA with methylation and ensures DNA is packaged appropriately (Amir et al. 1999). *MeCP2* also affects synaptic formation and hippocampal memory (Jiang et al. 2004; Li et al. 2012; Skene et al. 2010). Extensive histone acetylation increases are a consequence of this *MeCP2* mutation found in mice that are associated with increased stress, social withdrawal, and profound cognitive impairment (Shahbazian et al. 2002). Rett Syndrome is an example of how disrupted mC machinery affects the regulation of histone modifications, and thus gene expression and cognitive and social phenotypes. Another example of the manifest impact of histone modification variations in humans is the intellectual disability disorder Rubinstein-Taybi Syndrome (RTS) (Alarcón et al. 2004). RTS individuals have a mutation in their *CBP* gene, which transcribes for a protein that promotes histone acetylation and, thus, gene expression. Because of this mutation, RTS individuals and animal models of the disease present with profound cognitive impairments and substantial decreases in histone acetylation throughout the genome (Josselyn 2005; Kalkhoven et al. 2003; Korzus et al. 2004). In mouse models of RTS, the epigenetic action of *CBP* is disrupted specifically after separation of the pup from their mother (Wang et al. 2010). Taken together, these two mutations provide evidence for the weighty impact of epigenetic mechanisms in learning and the ensuing social consequences.

There is mounting evidence suggesting the importance of epigenetic modifications specifically affecting *BDNF* gene transcription, necessary for activity-dependent neuronal plasticity. Histone deacetylase 2 (HDAC2), methyl-cytosinephosphate-guanine–binding protein 2 (MeCP2), and DNA methyltransferase 1 (DNMT1) can substantially induce chromatin remodeling in the promoter regions of the *BDNF* gene, which can subsequently modulate hippocampal synaptogenesis and cognitive function (Chen et al. 1967; Kavalali et al. 2011). Transcriptionally, with neuronal activity there is also an increase in mRNA levels and consistent decrease in mC changes at *BDNF* in the CA1 hippocampal region after fear conditioning. Additionally, there is a reduction in the long noncoding antisense RNA that downregulates *BDNF* expression (Lipovich et al. 2012; Lubin et al. 2008). *BDNF* promotes local synaptic protein mRNA translation and plays a necessary role in neuronal development, synaptic plasticity, and learning (Minichiello 2009; Park and Poo 2013; Takei et al. 2004). Epigenetic differences affecting *BDNF* have been associated with early-life adverse experiences, early-life stress, neglectful parenting, depression, posttraumatic stress disorder, and may be a valid biomarker for some psychiatric diseases due to its unique role in their pathology (de Lima et al. 2011; Kang et al. 2013; Roth and Sweatt 2011a, b; Roth et al. 2011; Seo et al. 2016; Zheleznyakova et al. 2016).

Epigenetics acts as the synaptic connection between genes and the environment, even at the synapse itself (Boyce and Kobor 2015). While there is evidence that the prenatal environment affects epigenetic differences, the clear role of epigenetics in the encoding of long-term memories indicates the importance of differences in these mechanisms as a response to postnatal experiences as well. Thus, as expected, there is an abundance of examples in human and animal models of environmental exposures correlating with differences in epigenetic mechanisms, much like environmental exposures correlate with differences in neural architecture and behavior (Araki et al. 2016; Bedrosian et al. 2018; Boyce and Kobor 2015; Essex et al. 2013; Gassen et al. 2017; Klengel et al. 2013; Mcgowan et al. 2009, 2011; Turecki and Meaney 2016; Weaver et al. 2004). Even epigenetic clocks are sensitive to environmental differences throughout the lifespan (Jylhävä et al. 2019). Also, similar to neural development, early life social experiences can sometimes more strongly relate to epigenetic differences than adult experiences, possibly due to the critical period of development and learning occurring at that time (Boyce and Kobor 2015; Bush et al. 2018; Gassen et al. 2017; Klengel et al. 2013; Lam et al. 2012; Wagner et al. 2015). Using the theoretical framework that changes in neural architecture and genomic transcription are reactions to the environment, it naturally follows that these long-term adaptations would happen early in childhood development in order to increase the chance of survival in whatever positive or negative circumstances are present. Learning from the social environment is one mechanism of behavioral neurogenomics where changes to neuroanatomy, epigenetic modifications, and behavior work in concert to adapt to an external stimulus. There are multiple types of stimuli that can act as an impetus for this learning, such as social reward, pain, and stress. We will discuss each of these possible provocations in the relevant social environments.

4 Interpersonal Environments

4.1 Parenting and Enriched Environments

The first social environment a human infant is born into is the uniquely vital and interpersonal relationship with a caregiver. Human altricial young need constant care and affection, which they hopefully receive from their primary caregiver, often a parent. Having a positive, loving relationship in infancy with this person, who responds immediately and effectively to the child's needs, has lifelong effects (Zayas and Hazan 2015). Attachment theory, one of the most well-established social psychological theories, provides a foundation to understand both the impact and quality of early close relationships amid development.

In development, establishing successful relationships with adults and peers provides a foundation of capacities that children will use for a lifetime (Belsky and Cassidy 1994; Pietrornonaco and Barrett 2000; Thompson 2000; Zayas and Hazan 2015). Thus, Bowlby, father of attachment theory, referred to these attachment patterns as being "from cradle to grave" (Bowlby 1979, p. 129), possibly similar to the epigenetic modifications to be established in infancy and lasting through mortality. This attachment theoretical foundation provides a comprehensive account of the ontogeny and developmental sequelae of infant caregiver bonds, as well as a framework for investigating how perturbations of this system may result in individual differences. Additionally, animal models of pair bonding and advances in fMRI technology have contributed to a rich literature delineating the neural systems that underlie attachment behaviors (see Beckes et al. 2015 for review). These manifestations of social bonds in terms of physiology, emotion, and behavior are assumed to reflect the functioning of mental representations.

A defining principle of attachment theory is that past relationships and interactions with the social environment are stored in memory (e.g., Bowlby 1979, 1982; Bretherton and Munholland 2008; Collins et al. 2004; Pietromonaco et al. 2000; Zayas et al. 2011). Mental representations consist of detailed memories of interactions with, and conscious and nonconscious affective evaluations of, attachment figures (Zayas and Shoda 2005, 2015), as well as strategies to regulate negative affect in stressful and threatening situations (Collins et al. 2004; Pietromonaco et al. 2006; Zayas et al. 2009). From a psychological perspective, mental representations are impactful because they implicitly affect perceptions and expectations about likely events and patterns (Baldwin et al. 1993; Günaydin et al. 2012; Zayas et al. 2009). From a neuroscience perspective, these mental representations are akin to the engrams that are formed from a collection of neuronal connections built by LTP and recalled to regulate affect in times of need. Finally, from an epigenetic perspective, these mental representations may reflect the adaptations to early caregiver relationships and social environments that ultimately result in differing phenotypes. In regard to the later, there is a wealth of literature exhibiting reported epigenetic differences correlated with early caregiver relationships.

In animal research, the majority of studies have used rat models from the first 10 days of life, which represents a sensitive period in rat development known to facilitate early learning and infant–caregiver attachment (Roth and Sweatt 2011a, b). To continue the understanding of social epigenetics as adaptation in memories and the capacity for learning, one study found that poor early maternal caregiving in mice led to difficulties in spatial cognitive tasks (Bredy et al. 2004). More recent work in mice also found that expression of glutamate receptors necessary for LTP and LTD such as NMDA and AMPA in the ventromedial prefrontal cortex, the cognitive component of the socioemotional circuit, decreased in socially isolated adolescent, but not adult, mice (Lander et al. 2017). It has also been found that young mice which are not isolated, but receive less sensitivity and care from their mothers have abnormal hippocampal and amygdala *BDNF* gene expression necessary for learning and synaptic plasticity (Macrì et al. 2010). Similarly, amounts of BDNF decreased in the prefrontal cortex and hippocampus of juvenile socially isolated rodents (e.g., Branchi et al. 2004; Branchi 2009; Chatterjee et al. 2007; Choy et al. 2008; Fumagalli et al. 2007; Lippmann et al. 2007; Nair et al. 2007; Zimmerberg et al. 2009). These findings speak simultaneously to both the effect of social relationships on learning and the sensitive developmental period in which the plasticity of this adaptation takes place. In general, these findings are illustrative of the wider, rich literature on the epigenetic effects on plasticity in the prefrontolimbic and hippocampal regions of offspring exposed to reduced, or absent, maternal care (Branchi et al. 2006; Matas et al. 2016).

Stress effects also relate to a poor versus positive early caregiver relationship. An often-cited paper on the topic is by Weaver et al. (2004), on the relationship between maternal licking and grooming with mC differences in the promoter region of the glucocorticoid receptor gene (*NR3C1*). While they found that more licking and grooming from maternal rats lead to hypomethylation in the promoter region of the *NR3C1* and patterns of methylation in a broader surrounding area (Mcgowan et al. 2011), this study has yet to be replicated and is met with some skepticism in the field (Jones et al. 2018). However, more recent work in mice has also found different biological reactions to maternal caregiving in the hippocampus, a region associated with both learning and reactions to stress, and the dorsal raphe nucleus (DRN), the brain center for serotonin production and distribution (Araki et al. 2016; Bedrosian et al. 2018). In the DRN specifically, Araki and colleagues found hypomethylation affecting the GABA(B) receptor, which is a common pharmacological target for depression and anxiety relief (Araki et al. 2016; Felice et al. 2016).

Similar positive relationships between parental nurturance and memory development have also been found in humans (Farah et al. 2008). Generally, increased positive, engaged social environments increase memory formation in both humans and nonhuman primate models (Farah et al. 2008; Kozorovitskiy et al. 2005). Epigenetically, human studies have primarily focused on the mC of a few genes of specific and special prominence in the research, namely *BDNF* (see Zheleznyakova et al. 2016 for review), *NR3C1* (see Turecki and Meaney 2016 for review), *SLC6A4* (see Moore and Kobor 2018 for review), and *OXTR* (see Maud et al. 2018 for review). All of the proteins these genes encode have many functions across learning

and homeostatic change, especially in relation to neurodevelopment and stress. For example, the *BDNF* gene codes for brain-derived neurotrophic factor, the canonical neuronal growth factor in the brain widely involved in the formation of any neuroarchitectural changes. Additionally, *NR3C1* is the most widely researched gene in regards to fMRI and stress-causing environments, such as poor maternal care, in both the animal and human literature (Jones et al. 2018; Turecki and Meaney 2016). One recent study found that increased maternal responsiveness and touch were correlated with hypomethylation in *NR3C1* exon 1F in female children (Ostlund et al. 2016). This sex-dependent response has been replicated in other studies as well, such as Garg et al. (2018) finding the greatest DNA methylation differences among attachment styles in females. Additionally, they found that across the sexes, attachment behavior patterns were correlated to over 10% of global mC differences in children, suggesting large biological responses to the sensitivity and consistency of the parental care environment (Garg et al. 2018).

Another nuanced aspect of the caregiver relationship is soft touch, which is incredibly important in healthy, normative infant development (Barnett 2005). There were significant mC differences between children who received high amounts of soft touch and those who did not as infants. Additionally, infants who were more distressed, yet received lower amounts of touch, were epigenetically younger, possibly indicating a biological developmental delay (Moore et al. 2017). This is most likely due to the social buffering effects of both mental representations and human touch. For example, holding the hand of a stranger can reduce both the subjective experience of pain and its neural signature, but holding the hand of a close relationship partner reduces pain in these areas to an even greater extent (Coan et al. 2006).

Ultimately, the research indicates that having a healthy, supportive early social environment leads to positive epigenetic, neurological, and psychological outcomes. This is most clear when examining the literature on the benefits to enriched environments both in buffering stress and in rescuing memory formation. In humans, for example, having a supportive family environment during development protected against harmful cellular and epigenetic aging due to racial prejudice experiences. However, individuals without a supportive family environment did experience biological weathering (Brody et al. 2016). In animals, enriched environments including both positive caregivers as well as social play and peer interactions associated with global brain differences such as larger total cortical weight, especially the dorsal cortex, and larger ratio of cortex weight to overall brain weight (Rosenzweig and Renner 1986). Although less than some of the more negative aspects of development, there is a respectable amount of literature on the effects of this socially and cognitively enhanced environment in model animals. One such study found that, even with poor early caregiving, having an enriched environment rescued the gene expression of the NR2A and NR2B subunits of the NMDA receptor and AMPA receptor, both suggesting that poor early caregiving is associated in a reduction of learning potential through a reduction of NMDA receptor activity, and that an enriched environment with peer sociality and cognitive stimulation can combat these learning effects (Bredy et al. 2004).

This effect is most likely related to the demonstrated modulation of synaptogenesis by exposure to environmental stimuli (Eckert and Abraham 2010; Fischer et al. 2007; Ramírez-Rodríguez et al. 2014). Being housed in a communal nest as a juvenile, a form of enriched environment, is linked to increased sociocognitive functioning and increased expression of BDNF in rodents (Branchi 2009). In fact, in enriched environments with dynamic social play, overall memory function is improved in rats, and even exposure to chemically induced short-term memory impairment in neonatal rodents can at least be partially rescued through enriched environment alone with no other pharmacological interventions (Shen et al. 2013; Shih et al. 2012). The cognitive disruption of this chemical impairment has been shown to derive through epigenetic protein interactions including such proteins as histone deacetylase 2, methyl-cytosine-phosphate-guanine–binding protein 2, and DNA methyltransferase 1 in the *BDNF* promoter region inhibiting BDNF expression necessary for synaptogenesis during development, which are specifically attenuated by an enriched environmental intervention alone (Wu et al. 2016a). Even in aging mice, which had altered H3 histone acetylation and histone methylation in hippocampal tissue, intervention with an enriched environment improved long-term memory deficits by reversing histone methylation around the *BDNF* gene in rodent hippocampal tissue (Morse et al. 2015). This study also indicated that histone lysine methylation may be a necessary transcriptional mechanism by which environmental enrichment rescues memory formation replicating previous literature in young rodent populations (Kuzumaki et al. 2011). Another pathway through which enriched environment improves memory is by preventing epigenetic changes, especially mC and histone deacetylation, driving oxidative stress (Griñan-Ferré et al. 2016). These environments do not need to be lifelong to have strong effects either. Even a relatively brief exposure to an enriched environment including dynamic social stimulation in juvenile mice enhances LTP through a cAMP/p38 MAP kinase-dependent signaling cascade (Arai et al. 2009).

However, not all early relationships are positive experiences. For example, both maternal and paternal life stress during early life was correlated with adolescent differences in mC in humans (Essex et al. 2013). In rats, newborns exposed to a stress-abusive mother showed increased methylation in the promoter region, and decreased expression, of the *BDNF* gene (Huang and Reichardt 2001). This difference in BDNF concentrations for abused versus non-abused rats appears to persist through adulthood (Roth et al. 2009). In this same study, a different group of newborn rats was also exposed to positive caregiving mothers. Both the maltreatment and beneficial caregiving mothers initially caused an increase in *BDNF* mRNA levels in the hippocampus (Roth et al. 2009). Both experiences, regardless of valance, equitably guided the growth of new neuronal connections in the memory center of the brain. Adaptationally, negative relationships and social environments are just as powerful as positive social learning experiences.

4.2 Pain, Stress, and Trauma

In early care environments, the perception of safety is the most critical component (Porges 2011). Breaching this trust results in negative social experiences that can be both stressful and painful. There are many ways to experience pain, such as acutely, chronically, physically, and emotionally; in abusive early social environments, children are exposed to all four kinds of pain. Pain is such a powerful motivator for learning that is often used in animal models for fear conditioning, which is quick to establish and difficult to extinguish (Hermans et al. 2006). This particular form of learning has been found to require epigenetic modifications to take place and results in epigenetic differences (see Dias et al. 2015 for review). Thus, the epigenetic consequences of such early-life adversity are, undoubtedly, affected by learning and the physiological consequences of experiencing pain.

It is first important to establish that the literature indicates neural reactions to physical and socioemotional pain are exceptionally similar, specifically in regards to the affective processing of pain (Eisenberger and Lieberman 2004; Papini et al. 2015). However, though pain is processed in the same regions, socioemotional pain, such as social rejection or isolation, is more potent on a chronic timescale because it is much more easily relived and remembered than physical pain once the original source of pain has subsided (Meyer et al. 2015). Animal studies have also repeatedly found that any unpredicted reward devaluation, such as through a sudden or bewildering social rejection, triggers the brain circuits involved in pain and stress (Papini et al. 2015). Individuals who experience abuse, especially from a caregiver, may experience the physical pain of abuse, but most certainly experience the social pain of rejection and betrayal in that moment and for years later.

Pain is the hedonically aversive conscious experience of the nociception response to damage (Moseley and Butler 2015). When physical damage to peripheral tissues occurs, this initiates an immune cascade of inflammatory mediators (Benzon et al. 2011). For example, bradykinin produces inflammatory pain and hyperalgesia through activation of G-protein-coupled receptors. Additionally, cytokines such as tumor necrosis factor alpha and interleukin 6 are released to moderate the inflammatory process and promote pain signaling by sensitizing nociceptors (Benzon et al. 2011). While these inflammatory mediators directly cause pain as a signal of tissue damage, they also modify sensory neurons, amplifying pain signal during transmission to the spinal cord, additionally motivating the desire to minimize injury and remove the aversive stimulus. Through subsequent changes in reaction to this acute event, such as gene regulation, receptor expression, glial activation, and sensitization, this pain may be maintained to become chronic pain (Denk and McMahon 2012). This immune response alone may result in epigenetic modifications, especially as immune responses lead to different cell type compositions in the blood as well as differentiation in epigenetic markers through the adaptive immune response (Janeway 2001).

This immune response is an important aspect of the sensory discriminant pain pathway. The input from this pathway passes through the nerves, spine, and brain

stem to the thalamus and insular cortex where the homeostatic relevance and intensity of the signal is discerned (Craig 2003). However, there is also a learning aspect of pain through the formation of a threat memory. This is referred to as the affective motivational pain pathway and requires calcitonin gene-related peptide (CGRP) activity in the amygdala (Han et al. 2015). In fact, CGRP activity in the parabrachial nucleus, a junction between the cerebellum and brain stem, is both necessary and sufficient for pain responses and fear conditioning due to its role in transmitting pain information to the central amygdala (Han et al. 2015). The central amygdala, in turn, sends input to the anterior cingulate cortex, which governs the level of unpleasantness derived from the signal and the quality of the motivational response (i.e., the more unpleasant, the greater the aversive motivation) (Craig 2003). This affective motivational pain pathway is necessary for learning to avoid noxious and harmful stimuli in the future, whether those stimuli are physical or social, while the discriminant response is necessary for acute treatment of the damage in the present.

Both the discriminate and affective pain pathways show clear associations with epigenetic modifications, though most investigations have focused on evaluating potential affective pain mitigation (Odell 2018). For example, one investigation of chronic pain detected 1,147 genes with differing RNA expression enriched for pathways involved in neuronal development and cell differentiation (Alvarado et al. 2015). HDAC levels were shown to be increased in the spinal cord and of critical importance to the induction and maintenance of inflammatory hyperalgesia (Bai et al. 2010). Studies have also been performed demonstrating the efficacy of HDAC inhibitors in improving stress-induced visceral hypersensitivity (Cao et al. 2016; Maloney et al. 2015). Interestingly, although administration of HDAC inhibitors reduces mechanical and thermal hypersensitivity by half, this is only true when HDAC administration occurs preemptively (Denk et al. 2013). Histone methylation also changes with pain, such as the increase in expression of proinflammatory cytokine monocyte chemotactic protein 3 in response to pain correlating with a decrease of histone lysine methylation in that protein's gene promoter region (Imai et al. 2013). Additionally, a reduction of miRNA in the dorsal root ganglia significantly decreased pain-related gene transcription and inflammation, though the affective motivational response to pain was unaffected (Zhao et al. 2010).

DNA methylation differences have also been associated with pain exposure. The promoter region for cystathionine-beta-synthetase, an enzyme in the nociceptive signaling pathway, was demethylated and the protein upregulated when experiencing pain (Qi et al. 2013). A rodent model for neuropathic pain showed that chronic painful neuropathy led to global changes in the degree of mC in the brain. About 6 months following peripheral nerve injury, decreases in global mC were found in the prefrontal cortex and amygdala (Tajerian et al. 2013). In another rodent neuropathic pain model, increased methylation of the mu-opioid receptor gene proximal promoter in the dorsal root ganglion, a key region in pain processing, was demonstrated (Zhou et al. 2014). There is also evidence for miRNA regulation of opioid tolerance in this pathway (He et al. 2010). Mu-opioid receptors, especially, are integral to the pain pathways due to their ability to reduce the affective motivational

component of pain (Simons et al. 2014), even in the absence of any visceral tissue damage (Papini 2009).

Ultimately, pain biology is both a unique, intense, and specific immune response and learning experience. These epigenetic changes related to the experience and sensitization of the pain response, both through immune cascades and neural receptor modifications, should be considered as a possible consequence of socially and physically painful social environments. However, the benefit of pain's relationship to these systems is discovering potential protective environments as well. For example, in a rodent model, increased maternal licking and grooming associated with mC in the interleukin-10 gene correlate with an increase in expression in the nucleus accumbens reward center. This, in turn, increased interleukin-10 protein in nucleus accumbens glial cells and reduced mu-opioid receptor glial activation to exogenous opioids, which resulted in less drug abuse (Schwarz et al. 2011). The inextricable link between pain and learning allows other positive environments such as sensitive maternal care or an enriched social play environment to reduce pain and the necessity of the pain-mitigating pathway response.

The possibility of experiencing pain, because it is such a noxious experience, is highly motivating to mitigate or prevent injury whenever and wherever possible. This causes a combination of constant uncertainty and the need for hypervigilance – a recipe for chronic stress. Though intimately intertwined with the pain pathway, the stress pathway is rooted in the effects of uncertainty. Whereas fear and pain generally have specific and proximal stimuli that trigger these reactions, stress requires no such unique or immediately relevant stimulus. The stress response is the collection of immune, neural, and homeostatic mechanisms that shift into a long-term state of hyperawareness with the anticipation of threats that could appear at any time. While this state can be lifesaving when triggered appropriately, being in a constant state of fearful uncertainty is not a healthy ideal and has lasting deleterious biological effects.

The stress system is extraordinarily far-reaching and complex, but importantly to the current discussion, involves an interaction of epigenetic, neural, and behavioral responses through both the immune system and learning and memory. When there is uncertainty about a potentially aversive or harmful outcome, the downstream stress response is activated by the hypothalamus. The hypothalamus secretes corticotropin-releasing hormone (CRH), which stimulates release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn signals two different stress pathways, one fast and one slow (Gunnar and Quevedo 2007). The two major stress pathways are the sympathetic-adrenomedullary (SAM) response and the hypothalamic-pituitary-adrenocortical (HPA) response (Gunnar and Quevedo 2007). In the fast, immediate SAM response, the ACTH triggers the adrenal medulla to release norepinephrine and epinephrine, neurotransmitters required to cause a rapid and intense nervous system response and hypervigilant attention (Benarroch 2007). In the slower, long-lasting HPA response, ACTH triggers the adrenal cortex to signal the release of the stress hormones glucocorticoids, the most important of which is cortisol, thus

dysregulating metabolism, suppressing the immune system, and disrupting homeostasis through glucocorticoid receptor binding systemically (Gunnar and Quevedo 2007). During the stress response, homeostatic mechanisms attempt to maintain equilibrium over a wide range adaptive circumstances in order to respond to any possible challenge. Stress is, in essence, a "ready" state from which a large, quick biological response is primed at a moment's notice and equipped with constant vigilance. Therefore, this cascade of biological effects both elicits a physiological and behavioral response, and poises the requisite systems for future environmental reactivity. Unfortunately, when chronically stressed, there is a burden placed on these biological systems such as the immune system and metabolism that becomes harmful, referred to as allosteric load (Gunnar and Quevedo 2007; Gunnar 2017). An excellent theoretical understanding of this facet of the stress response system is to return to the gAP (Clayton 2000; Clayton et al. 2019). The brain has a response to an environmental stressor that leads to transcriptional and epigenetic changes. These changes then trigger a "neuroendocrine action potential" as this neural response triggers both immediate and long-lasting changes throughout the limbic system and multiple organs (Clayton et al. 2019).

The clear, widespread effect of stress throughout all physiological systems makes it an understandable and unique candidate for understanding the epigenetics of social environment. Many adverse environments, both in the interpersonal, such as traumatic and abusive relationships with caregivers and peers, and the societal, such as minority stress and socioeconomic status stress, can trigger these same underlying processes, as all are sources of aversive, potentially harmful uncertainty. It is then understandable why the most commonly researched gene in social epigenetics is *NR3C1*, the glucocorticoid receptor gene (Turecki and Meaney 2016). In Turecki and Meaney's study (2016), they found consistent mC at the *NR3C1* in exon 1F/17 regarding parental stress, but inconsistent in other types and later life stressors. Additionally, recent work found that stress affected mRNA methylation in a region-specific manner, ultimately altering fear learning and synaptic plasticity (Engel et al. 2018).

The literature does appear to indicate that chronic stress in early life has a greater impact on mC patterns than those that occur in later life, but more research in this area is needed to make a definitive statement (Austin et al. 2018; Esposito et al. 2016). Work on cumulative stress, as opposed to early life or later life considered separately, also indicates an association with accelerated epigenetic aging (Zannas et al. 2015a). If this stronger association with stress and mC at younger ages is robust, it may be due to differences in the immune system's environmental sensitivity during early development (Miller et al. 2011). Additionally, a key component of the attachment relationship is to learn and support affect regulation (Hazan and Shaver 1987); having a responsive, consistent caregiver helps children express and deal with their negative emotions without triggering the endocrine stress response as if an uncertain threat had been detected; however, in abusive relationships, the parent is the source of uncertainty and danger (Repetti et al. 2014). While it is true that a recent, large study in humans did not replicate findings from smaller studies that

reported correlations between mC patterns and chronic social stress (Marzi et al. 2018), this may be due to differences in sample size, population, and consistency among ecologically valid measures of the type, context, and experience of stress. More replications with standardized measures and large sample sizes are needed to make any definitive statements about detectable mC differences among those who have experienced chronic stress.

In addition to *NR3C1*, there is a robust literature associating early-life adversity such as social, physical, or parental stressors, with epigenetic changes in *BDNF* gene expression (Roth and Sweatt 2011a, b). These differences may correlate with a reduction in socioemotional learning and plasticity, and have shown an increased capacity for fear learning (Dias et al. 2015). Impacted learning has also been implicated in more specific associations than general stress, such as the several reported associations between trauma, abuse, and differences in epigenetic modifications (e.g., Dickson et al. 2018; Klengel et al. 2014; Lutz and Turecki 2014; Mehta et al. 2013; Roberts et al. 2018; Suderman et al. 2014; Weder et al. 2014) as well as associations specifically with posttraumatic stress disorder (see Zannas et al. 2015b for review). Though there is a range in the findings of epigenetics in regards to early life adversity from both candidate gene approaches and EWAS approaches, overlapping genes associated with stress, pain, learning, and the immune system were common. For example, one study found immune cell differences and accelerated epigenetic age associated with lifetime PTSD severity (Rutering et al. 2016).

It is possible that trauma and pain in early life leads to learning and adaptation to a harsher world that requires more vigilance instead of a conservative homeostasis, a molecular push toward fear conditioning instead of socioemotional development, and a greater sensitivity to pain in order to more quickly identify threats. Those who develop in positive, enriched environments, on the other hand, thrive with reduced allosteric load and have resilience that seems especially prominent in stress coping and synaptic plasticity. Instead of being able to learn more and put their energy toward other endeavors, these adaptations in a world of agonizing uncertainty could be primed or activated by epigenetic modifications for the sole purpose of survival. A plausible model of chronic life stressors proposes a similar line of reasoning and theorizes with significant evidence that epigenetic modifications set into motion by the cascade of stress hormones both affect and prime a traumatized individual for accelerated aging and biological weathering (Gassen et al. 2017). Supporting this hypothesis, a high number of sites used in mC epigenetic clocks are located within glucocorticoid response elements (Zannas et al. 2015a).

However, though it may not undo the harmful developmental environment, social support, especially touch, has been associated with stress buffering in many studies (Coan et al. 2006; Cohen and Wills 1985; Matthews and Gallo 2011; Ozbay et al. 2007, 2008). This is most likely through the dual mechanisms of affect regulation through a social buffer so as to not trigger the stress hormone cascade, and through the mu-opiates that are released in social reward reducing affective motivational pain in the nociceptive pathways and the intensity of perceived threats (LaGraize et al. 2006; Troisi et al. 2011). This may be why we see rescue effects of social, enriched environments for memory deficits as discussed above. This may also contribute to

the difficulty in reproducing many social epigenetic findings, as adverse effects are often accounted for, but buffering and resiliency effects are not.

5 Societal Environments

5.1 Minority Status and Socioeconomic Status

Chronic stress has clear physiological, psychological, neurological, and epigenetic effects. However, not all chronic stress stems from abuse or trauma. In fact, not all chronic stress stems from interpersonal relationships at all. As a social species, we have developed a society with biases, prejudices, and hierarchies. Our modern social environments remain embedded by historical power relationships. Though there is much work being done to correct these injustices, racial, ethnic, gender, sexuality, or religious minorities, as well as those with a low socioeconomic status (SES) and social position, face considerably more stress from societal pressures and inequities than their counterparts. This stress may then exacerbate these inequalities through cellular means, as well as societal.

A minority group that is illustrative of how social structures can affect epigenetics and, ultimately, associate with extreme behavioral phenotypes are people living with schizophrenia. Schizophrenia is a mental illness often characterized by flat affect, hallucinations, and psychosis for which the polygenetic burden is significantly associated with epigenetic variation, suggesting that regulatory variation of the disorder stems from both the genome and environment (Cromby et al. 2019). Schizophrenia is the most thoroughly studied psychiatric disorder from an epigenetic perspective, mostly likely due to the clear environmental effects in its presence, onset, and trajectory. Specifically, there is evidence associating schizophrenia presence and onset with low SES, ethnic differences and racial discrimination, immigration, urban living, childhood adversity and trauma, and parental absence (Cromby et al. 2019). Though there is an underlying genetic component to the disorder clear from family clustering, this risk is compounded by exposure to these social environmental factors, such as minority status (Hutchinson et al. 1997). There is significant evidence that minority status, or seeming apart from society in any way, increases the likelihood of developing schizophrenia most likely due to the chronic societally based stress of social position (Bourque et al. 2011; Cromby et al. 2019; Selten 2005; Van Os et al. 2010). For example, Black Caribbean immigrants to the UK who grew up in the Caribbean do not express any increased risk of psychosis; however, their children, born and raised as Black Caribbean immigrants in the UK, had a seven times higher risk of psychosis (Hutchinson et al. 1997). This was familial risk within an ethnically homogenous sample, pointing toward a significant association with the chronic stressors of immigrant and racial minority status being significant drivers of schizophrenia risk. Even more physical variables, such as urban neighborhood residence,

that have been found to increase psychosis risk, appear to be more driven by social fragmentation than physical environment (Zammit et al. 2010).

Often compounding the chronic stress associated with minority status is the stress associated with class and SES. Most likely due to many social, physical, and biological factors encompassed in SES, there is a plethora of associations between SES and epigenetic changes. As SES is societally constructed, it is difficult to create an ecologically valid animal model with which to investigate epigenetic modifications; therefore, the majority of epigenetic research is focused on mC associations in humans. Low SES, especially during youth, has a significant and robust association with age acceleration and sites connected to immune function, development, and age-related diseases (Austin et al. 2018; Bush et al. 2018; Chen et al. 2011; Fiorito et al. 2017; Lam et al. 2012; McCrory et al. 2019; Mcdade et al. 2019; Simons et al. 2016; Tehranifar et al. 2013). Even with low-SES youth, greater self-control associates with improved socioemotional functioning and general success, but also epigenetic age acceleration, supporting the idea that increased allosteric load may contribute to worse health outcomes among the disadvantaged (Miller et al. 2015).

Another aspect of the relationship between SES and epigenetic modifications is that lower SES correlates with both smoking and drinking behavior (Sweeting and Hunt 2015; Van Oers et al. 1999). Not surprisingly, social stress also triggers the urge to smoke and drink (Fouquereau et al. 2003; Niaura et al. 2002). There is evidence that drinking increases emotional experiences and smoking temporarily reduces arousal as evidenced by reduced neurological event-related potentials, supporting a self-medication hypothesis of legal drug use (Choi et al. 2015; Sayette 2017). This relationship of SES with smoking and drinking, possibly as a way to deal with stress, may exacerbate epigenetic disparities due to the strong, reproducible effects of smoking and drinking on mC, especially on sites related to age, immune, and cardiovascular function (Brückmann et al. 2017; Goldowitz et al. 2014; Hillemacher et al. 2008; Mahnke et al. 2017; McCartney et al. 2018; Ponomarev et al. 2017; Tulisiak et al. 2017).

In addition to smoking and drinking, it is important in every mC investigation to account for cell type proportions, as these are the primary drivers of variation, but this is especially true in explorations of SES due to the significant immune system effects of the chronic stress system. For example, one study found that leukocyte composition of peripheral blood covaried with patterns of mC at many sites and mC was strongly associated with the monocyte inflammatory response (Lam et al. 2012). Monocytes also epigenetically aged faster in those exposed to low SES in early life (Austin et al. 2018). These immune responses, most likely from stress, may contribute, at least in part, to the association of SES, especially early life SES, with epigenetic age acceleration and aging-related disease risk, even controlling for related factors such as smoking and drinking (Austin et al. 2018; Simons et al. 2016). When Simons et al. (2016) investigated the main environmental driver of epigenetic age acceleration in a low SES sample, they found that it was the stress of financial insecurity that drove the SES and accelerated aging association, providing further evidence for the link between early life stress, immune response, epigenetic change, and health outcomes (reviewed in Miller et al. 2011). However, similar to other early exposures to

stress, the effects of low SES on immune response can be buffered through social support in the form of warm, positive caregiving (Chen et al. 2011).

5.2 *Social Effects on Physical Environment*

Though it may not be interpersonal or even seem entirely social, there is a social environment in the construction of our societies in the types of nutrients we can access, the configuration of neighborhoods in which we live, the services to which we have access, and the physical environments to which we are exposed. Both physical and social environments can affect our epigenetic modifications (Mcdade et al. 2017). The social administration of our physical environments is yet another form of social environmental influence to which we are able to learn and adapt psychologically and biologically, whether it is conscious or unconscious.

One major aspect in the construction of socially administered environments is the spatial sorting of people based on their SES, race, or ethnicity. As discussed above, there is evidence chronic stress that accompanies being in a reduced societal position, whether through racism or classism, as well as the stress of deprivation, associates with epigenetic change. However, in addition to this divide, health differences among neighborhoods persist even after adjusting for SES and demographic factors, most likely due to the impact of broad environmental factors such as access to nutrition or exposure to pollution (Mair et al. 2008; Paczkowski and Galea 2010; Pickett and Pearl 2001; Roux and Mair 2010). Factors linked to differences in physical environment most likely contribute to and reinforce the detrimental effects of chronic societal stress on low SES and minority communities (Bleich et al. 2012; LaVeist et al. 2011). Unsurprisingly then, physical environment and location are also tied to risk of schizophrenia (March et al. 2008).

One example of how physical environments may perpetuate the biological differences among classes and races are food deserts. These are areas, either particularly urban or rural, where fresh produce and other healthy foods are either not available or too expensive to be purchased as an everyday source of caloric intake. Low SES neighborhoods are especially likely to be located in a food desert (Ghosh-Dastidar et al. 2014). Food availability and food advertising, which is different for lower SES neighborhoods, influence energy intake and the nutritional value of foods consumed (Grier and Kumanyika 2008; Harris et al. 2009). The wealth of literature on the epigenetics of nutrition, especially prenatal nutrition, pales only in comparison to epigenetic work in cancer (Anderson et al. 2012). The importance of a balanced, healthy diet from conception and throughout life on epigenetic modifications is an incredibly robust finding, as are similar results for morbidity and mortality (e.g., Anderson et al. 2012; Gabbianelli and Damiani 2018; Lillycrop and Burdge 2012; Mathers 2006; Milagro et al. 2013; Navarro et al. 2017; Zhang 2015). Along similar lines, the structure of a socially administrated physical environment can also be linked to differences in children's physical activity (Bringolf-Isler et al. 2010; Davison and Lawson 2006; Galvez et al. 2010; Sallis and Glanz 2006). Physical activity is often

linked to morbidity and mortality, as well as epigenetic modifications, learning, and aging (e.g., Kaliman et al. 2011; Kashimoto et al. 2016; Kirchner et al. 2013; Ling and Rönn 2014; Mikkelsen et al. 2017; Moylan et al. 2013; Rodrigues et al. 2015; Zimmer et al. 2016). Another example is physical proximity to hazardous sites and pollution, which tend to be more prevalent in low-income or minority neighborhoods (Brulle and Pellow 2006; Mohai et al. 2009; Morello-Frosch et al. 2011). The effects of exposure to air pollution are well evidenced in both morbidity and epigenetics research (e.g., Barouki et al. 2018; Brook et al. 2010; Chen et al. 2016; Clifford et al. 2017; Gref et al. 2017; Laumbach and Kipen 2012; Luyten et al. 2018; Mustafíc et al. 2012; Rider et al. 2016; Somineni et al. 2016; Tzivian 2011). This is most likely due to the immune responses to breathing in toxic exogenous factors (Tzivian 2011).

Additionally, neighborhood conditions can create stress, such as feeling unsafe, as well as acting as social buffers against adverse effects of stress such as social cohesion or integration into the neighborhood or environments such as work or school (Cutrona et al. 2006; De Silva et al. 2005; Do et al. 2011; Mair et al. 2008; Merkin et al. 2009). One possibility of why some immigrant groups have better morbidity and mortality than other groups in the same city is the social support and cohesion within the community (Matthews et al. 2010). As discussed above, the stress response affects many systems and may lead to widespread epigenetic modifications, especially during early development. Once again, even the midst of possible adaptation to a harmful environment, a positive, enriched social environment shows ecological rescue effects for health. Our epigenetic mechanisms modify, our neurological mechanisms encode, and our psychological mechanisms learn from our social environments.

6 Conclusion

From the moment we are born, our social relationships are a key component of how the world affects us. They are one of the first postnatal inputs afforded to the rapidly developing neonatal biology and are essential for survival. While data on epigenetics and the social environment have been spread out across disciplines, one can imagine potential examples for stringing these findings together cohesively. One example of this cell-to-society effect could be a child born in a family with insensitive caregivers who consistently do not respond to the child's needs or give contact comfort. This, in turn, could lead to the hypermethylation of the promoter region for the *BDNF* gene, less *BDNF* transcription, and less BDNF present in the hippocampus and socioemotional circuit of the brain. This would then decrease the ability of the child to learn from their social environment and affect their ability to have successful interpersonal relationships. This lack of social efficacy could then make it more likely the individual would experience social pain and more difficult to receive social support to buffer stressful experiences throughout their lives. Without social buffering, the stress response could be triggered more often, resulting in reduced immune responses and homeostatic compensation through epigenetic modifications.

Over time, these cumulative biological and behavioral responses could increase allosteric load and possibly lead to accelerated aging and health decline, which may also affect access to services, financial earning ability, and physical environment to further exacerbate biological and sociological disparities seen at a population level.

These social environments are an opportunity for adaptation through experience seen pandiscipline through concepts such as mental representations, LTP and memory engrams, and epigenetic change such as histone lysine trimethylation affecting the promoters of synaptic plasticity-related genes. Fundamentally, all psychological, neurobiological, and epigenetic reactions to these social environments are opportunities to learn from and adapt to them in order to best thrive in the world as it is, whether that environmental situation be ideal, violent, or deprived. The experience-dependent plasticity gained from the interaction between neuroscience and epigenetics is integral to this adaptation (Clayton et al. 2019). These systems may be working as a new state of vigilance. Although the context of the social environment is paramount in the specific reaction and modification, the ultimate goal and underlying mechanistic interplay remain largely the same – to learn from our social world to better survive in the environment we find ourselves in.

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The Role of Dynamic Histone Modifications in Learning Behavior

Andre Fischer

Contents

Abstract Epigenetic mechanisms have been linked to memory formation under physiological and pathological conditions. Therapeutic strategies that target the epigenetic machinery have been successfully used in preclinical studies investigating cognitive phenotypes linked to neuropsychiatric and neurodegenerative diseases. This chapter will specifically discuss the role of histone modification in the adult brain with a focus on learning and memory processes in the healthy and diseased brain. Data on dynamic changes in histone modification during memory processes as well as the most current knowledge on the corresponding enzymatic machinery in the adult brain will be summarized and discussed in the context of potential therapeutic opportunities to treat brain diseases.

Keywords Epigenetic therapies · Histone methylation · Learning · Memory · Neurodegenerative diseases · Neuroepigenetics · Neuropsychiatric diseases

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1 Introduction: Neuro-epigenetics and Histone Modifications

The tight control of gene expression programs is essential for cellular function. In turn, deregulated gene expression has been implicated with the pathogenesis of various diseases. In addition to the activity of transcription factors, epigenetic mechanisms are key processes that control gene expression at a systems level (Allis et al. 2007a). The term epigenetics has been originally introduced by Conrad Waddington to describe heritable changes of a phenotype that do not depend on altered DNA sequence (Waddington 1953). In addition to DNA methylation and the action of noncoding RNAs, a central epigenetic process is the modifications of histone proteins. The four core histones (H) build the nucleosome around which 147 bp of DNA is wrapped. The histone tails are subjected to posttranslational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, etc. (Vaquero et al. 2003) which are mediated by the counteracting activities of enzymes that add or remove histone modifications and are thus called "writers" and "erasers." For example, histone acetylation is mediated by histone acetyltransferases (HAT, writers) and histone deacetylases (HDAC, erasers). The activity of such enzymes is believed to give rise to a combinatorial pattern of chromatin modifications, the so-called histone code (Strahl and Allis 2000). Such histone modifications are recognized by proteins that subsequently affect cellular processes such as gene expression and are referred to as chromatin "readers" (Fischer 2014a). The field of neuro-epigenetics investigates the abovementioned epigenetic processes in the context of neuronal plasticity, memory function, and brain diseases (Fischer 2014a; Day and Sweatt 2011; Jakovcevski et al. 2013b).

Various cellular mechanisms have been linked to memory function, and tightly controlled dynamic changes in gene expression are one requirement to enable the consolidation of long-term memories (Flexner et al. 1962, 1963; Davis 1984; Igaz et al. 2002; Frey and Morris 1998; Moncada et al. 2011; Fischer 2014a). In addition, deregulated gene expression is seen in brain diseases linked to memory impairment (Lu et al. 2004; Liang et al. 2010; Ginsberg et al. 2010; Twine et al. 2011; Blalock et al. 2011; Kim et al. 2012a; Arefin et al. 2012; Caldeira et al. 2013; Mills et al. 2013). Understanding the mechanisms that orchestrate gene expression programs in brain cells is thus of utmost importance, and epigenetic processes have emerged as key players that enable synaptic plasticity and memory formation.

A number of excellent review articles have discussed the role of epigenetic processes such as DNA methylation and noncoding RNAs in synaptic plasticity, memory function, and brain diseases (Day and Sweatt 2010; Fiore et al. 2011; Fischer 2014a; Jakovcevski and Akbarian 2012; Campbell and Wood 2019). This chapter will focus on recent findings on the role of histone modifications in memory formation and cognitive diseases.

1.1 Histone Modifications and Memory Consolidation in Health and Disease

First evidence that histone acetylation might play a role in memory consolidation stems already from 1979. Using C14-labeled acetate, Schmitt and Matthies could show that acetylation of histones is altered when rats are subjected to a learning paradigm that leads to the formation of long-term memories (Schmitt and Matthies 1979). Only at the beginning of this century, such studies were followed up, and a number of labs could demonstrate that memory formation in rodents is correlated to increased activity of HATs (Swank and Sweatt 2001) and changes in histone acetylation that were measured by semiquantitative immunoblot analysis using antibodies specific to acetylated lysine residues of histones (Levenson et al. 2004; Fischer et al. 2007; Bousiges et al. 2010). These methods are able to detect changes in bulk levels of histone acetylation but do not allow for any deeper insight to genes regulated by changes in histone acetylation. Such issues were circumvented when researchers started to perform chromatin immunoprecipitation (ChIP) followed by either qPCR or later next-generation sequencing approaches (ChIP-seq) to study histone acetylation at the single gene or genome-wide level (Peleg et al. 2010). An important consideration in this context is the complexity of cell types in the brain. Technological advances now also allow the analysis of histone modifications via ChIP-seq at the single-cell level, but these methods have not been applied yet to the neurosciences (Grosselin et al. 2019). Most researchers nowadays perform cell type-specific analysis of histone acetylation via ChIP-seq using FACS-sorted nuclei that distinguish at least between neuronal and non-neuronal cells (Shulha et al. 2012; Benito et al. 2015; Halder et al. 2016), which is of utmost importance for the analysis of brain diseases characterized by neuronal cell death in combination with inflammatory processes.

Thus, there is now substantial evidence confirming the initial observation by Schmitt and Matthies that changes in histone acetylation but also other histone modifications are observed in response to learning stimuli or during the pathogenesis of brain diseases. Moreover, there is now convincing evidence from the analysis of mutant mice or other model systems that these epigenetic processes play a key role in cognition. In the following we will discuss these data in detail.

1.2 Histone Acetylation

As discussed above, early findings reported altered histone acetylation in response to memory training, and in most cases, these changes were linked to active gene expression, hence an increase of histone marks linked to active gene expression such as histone acetylation at various lysine residues of H3 and H4 (Levenson et al. 2004; Alarcon et al. 2004; Fischer et al. 2007; Miller et al. 2008; Federman et al. 2009; Peleg et al. 2010; Dagnas and Mons 2013; Fischer 2014a). These data led to

the hypothesis that inhibition of HDAC proteins might increase memory formation, which was confirmed in multiple studies (Alarcon et al. 2004; Levenson et al. 2004; Wood et al. 2005; Fischer et al. 2007; Fischer 2014b). As a consequence HDAC inhibitors were tested in models for cognitive diseases such as Alzheimer's disease but also other cognitive diseases, and indeed administration of HDAC inhibitors was found to ameliorate memory impairments and cognitive phenotypes in numerous studies (Fischer 2014a, b). Some of the HDAC inhibitors such as vorinostat are already used in patients for other indications, and it should be mentioned that oral administration of vorinostat could ameliorate memory impairment and deregulated transcriptome plasticity in various mouse models for age-associated memory impairment and Alzheimer's disease (Kilgore et al. 2010; Benito et al. 2015). Thus, this drug is currently tested in Alzheimer's disease patients (https://clinicaltrials.gov/ct2/show/NCT03056495).

Histone acetylation is generally linked to active gene expression, and thus initially not much attention was paid to potential difference of, for example, learning-induced increases in H3K9ac, H4K14ac, or the H4 acetylation sites H4K5, H4K8, H4K12, and H4K12, and often histone acetylation was assayed using pan-antibodies that would detect all of the abovementioned H3 and H4 acetylation sites. While this is still a valid approach, there is also evidence that H3 and H4 acetylation sites might serve specific cellular functions. For example, the onset of cognitive decline during aging was linked to deregulated acetylation of H4K12 (H4K12ac). While memory training induced a transient increase in various histone acetylation marks in the hippocampus of young and aged mice, H4K12ac did not respond anymore to such a stimulus in old animals. When ChIP-Seq was combined with transcriptome analysis, the data revealed that H4K12ac is critical for the initiation of a learning-induced gene expression program via a mechanism that involved transcriptional elongation (Peleg et al. 2010). These data are interesting since the chromatin reader proteins BRD2 and BRD4, which belong to the BET (Bromodomain Extraterminal) subfamily of chromatin readers, were found to specifically recognize H4K12ac. Similar to HDAC inhibitors, the BRD2/BRD4 inhibitor JQ1 was found to improve memory function in wild-type mice and in a mouse model for Alzheimer's disease (Benito et al. 2017).

All of these data are summarized in a number of excellent review articles (Fischer 2014a, b; Deussing and Jakovcevski 2013) and inspire the interested to better understand the enzymatic machinery that controls histone acetylation.

The mammalian genome encodes for at least 18 HATs that are subdivided into the GNAT (*G*cn5 *N-a*cetyl*t*ransferases) family, the MYST (*MOZ*, *Y*bf2/Sas3, *S*as2, *T*IP60) family, the p300/CBP family, the SCR family nuclear receptor coactivators, and the several other HATs that cannot be grouped into a certain family (Lee and Workman 2007; Allis et al. 2007a). Most of these proteins are expressed in the mouse and human brain (Table 1). The best-studied HATs in the context of mammalian brain function belong to the p300/CBP family. Multiple studies demonstrated a role for CBP/*KAT3A* in memory consolidation (Alarcon et al. 2004; Korzus et al. 2004; Wood et al. 2005, 2006; Chen et al. 2010; Barrett et al. 2011). It should be mentioned at this point that memory function is routinely analyzed in rodents via a

Table 1 Regulators of histone acetylation and methylation in learning and memory

Enzyme	Catalytic action	Linked to memory-related processes
Histone acetyltransferases		
KAT1A (HAT1)	Histone acetylation	No conclusive data
KAT2A (GCN5)	Histone acetylation	Yes, loss of KAT2A impairs memory
KAT2B (PCAF)	Histone acetylation	Yes, loss of KAT2B impairs memory
KAT3A (CBP)	Histone acetylation	Yes, loss of KAT3A impairs memory
KAT3B (P300)	Histone acetylation	Yes, loss of KAT3B impairs memory
KAT4 (TAF1)	Histone acetylation	No conclusive data
KAT5A (TIP60)	Histone acetylation	Yes, loss of KAT5A impairs memory
KAT6a (MYST3)	Histone acetylation	No conclusive data
KAT6B (MYST4)	Histone acetylation	No conclusive data, linked to neurogenesis
KAT7 (MYST2)	Histone acetylation	No conclusive data
KAT8 (MYST1)	Histone acetylation	No conclusive data
KAT9 (ELP3)	Histone acetylation	No conclusive data
KAT12 (GTF3C4)	Histone acetylation	No conclusive data
KAT13A (NCOA1)	Histone acetylation	Yes, loss of KAT2B impairs memory
KAT13B (NCOA3)	Histone acetylation	No conclusive data
KAT13C (NCOA2)	Histone acetylation	No conclusive data
KAT13D (CLOCK)	Histone acetylation	No conclusive data, circadian rhythm
Histone deacetylases (zinc-dependent, excluding sirtuins)		
HDAC1	Histone deacetylation	Yes, HDAC1 affects memory
HDAC2	Histone deacetylation	Yes, loss of HDAC2 improves memory
HDAC3	Histone deacetylation	Yes, loss of HDAC3 improves memory
HDAC4	Histone deacetylation	Yes, HDAC4 affects memory
HDAC5	Histone deacetylation	Yes, loss of HDAC5 impairs memory
HDAC6	Histone deacetylation	Yes, loss of HDAC6 improves memory
HDAC7	Histone deacetylation	No conclusive data
HDAC8	Histone deacetylation	No conclusive data
HDAC9	Histone deacetylation	No conclusive data
HDAC10	Histone deacetylation	No conclusive data
HDAC11	Histone deacetylation	No conclusive data

(continued)

Table 1 (continued)

Enzyme	Catalytic action	Linked to memory-related processes
Histone methyltransferases		
KMT2A (Mll1)	H3K4 mono-, di-, and tri-methylation	Yes, loss of KMT2A impairs memory
KMT2B (Mll2)	H3K4 mono-, di-, and tri-methylation	Yes, loss of KMT2A impairs memory
KMT2C (Mll3)	H3K4 mono-, di-, and tri-methylation	No conclusive data
KMT2D (Mll4)	H3K4 mono-, di-, and tri-methylation	No conclusive data
SETD1A	H3K4 mono-, di-, and tri-methylation	No conclusive data
SETD1B	H3K4 mono-, di-, and tri-methylation	No conclusive data
KMT1A (SUV39H)	H3K9 di- and tri-methylation	No conclusive data
KMT1B (SUV39H2)	H3K9 di- and tri-methylation	No conclusive data
KMT1C (G9A/EHMT2)	H3K9 mono- and di-methylation	Yes, loss of KMT1C impairs memory
KMT1D (GLP/EHMT1)	H3K9 mono- and di-methylation	Yes, inhibition of KMT1D affected memory
KMT1E (SETDB1)	H3K9 mono- and di-methylation	No conclusive data
KMT8A (PRDM2)	H3K4 mono-, di-, and tri-methylation	No conclusive data
KMT6A (EZH2)	H3K27 mono-, di-, and tri-methylation	Yes, loss of KMT6A impairs memory
KMT6B (EZH1)	H3K27 mono, di-, and tri-methylation	No conclusive data
KMT3F	H3K4 and H3K27 mono-, di-, and tri-methylation	No conclusive data
KMT3A (SET2)	H3K36 mono- di- and tri-methylation	No conclusive data
KMT3B (NDS1)	H3K36 di- and tri-methylation	No conclusive data
KMT3C (SMYD2)	H3K36 di- and tri-methylation	No conclusive data
KMT4 (Dot1)	H3K79 mono-, di-, and tri-methylation	No conclusive data
KMT5A (SET8)	H4K20 mono-methylation	No conclusive data
KMT5B (SUV4-20H1)	H4K20 mono-, di-, and tri-methylation	No conclusive data
KMT5C (SUV4-20H2)	H4K20 mono-, di-, and tri-methylation	No conclusive data
Histone demethylases		
KDM5A (JARID1A)	H3K4 demethylation	No conclusive data
KDM5B (JARID1B)	H3K4 demethylation	No conclusive data
KDM5C (JARIC1C)	H3K4 demethylation	Yes, loss of KDM5C affects memory

(continued)

Table 1 (continued)

Enzyme	Catalytic action	Linked to memory-related processes
KDM5D (JARID1C)	H3K4 demethylation	No conclusive data
KDM2A (JHDM2A)	H3K9 demethylation	No conclusive data
KDM4A (JMJD2A)	H3K9/H3K36 demethylation	No conclusive data
KDM4B (JMJD2B)	H3K9/H3K36 demethylation	Yes, loss of KDM5C impairs memory
KDM4C (JMJD2C)	H3K9/H3K36 demethylation	No conclusive data
KDM4D (JMJD2D)	H3K9/H3K36 demethylation	No conclusive data
KDM4E (JMJD2E)	H3K9/H3K36 demethylation	No conclusive data
KDM1 (LDS1)	H3K4 and H3K9 demethylation	Yes, inhibition of KDM1 impairs memory
KMD6A (UTX)	H3K27 demethylation	Yes, loss of KDM6A impairs memory
KDM6B (JMJD3)	H3K27 demethylation	No conclusive data
KDM7B	H3K27 demethylation	No conclusive data
KDM2A (JHDM1A)	H3K36 mono- and di-demethylation	No conclusive data
KDM2B (JHDM1B)	H3K36/79 mono- and di-demethylation	No conclusive data
KDM8 (JMJD5)	H3K36 mono- and di-demethylation	No conclusive data

number of well-established memory tests that assay different cognitive domains such as spatial reference memory or associative fear learning. Loss of CBP/KAT3A does not affect all types of memory, and specifically spatial reference learning in the hippocampus-dependent Morris water maze is only medley affected in mutant mice (Josselyn 2005). In line with these observations, experiments in which CBP/KAT3A agonists were administered to mice during spatial reference learning did not improve the consolidation of memories that was measured 48 h after training but improved the consolidation of remote memories when analyzed up to 16 days after the training (Chatterjee et al. 2013). The role of CBP/KAT3A in neuronal plasticity has been linked to its potential to regulate the expression of specific genes via the modulation of histone acetylation. For example, its effect on memory consolidation was linked its ability to induce the expression of nuclear receptor 4a1 (Nr4a1) (Vecsey et al. 2007; McNulty et al. 2012). However, detailed genome-wide analysis of transcriptional networks associated with CBP function in the adult brain is sparse, and gene array approaches thus far linked CBP function to calcium signaling, transcription and synaptic plasticity (Chen et al. 2010), and deregulated gene expression in response to environmental enrichment training (Lopez-Atalaya et al. 2011). Evidence for a role of P300/KAT3B in memory function stems for work showing that genetic reduction of P300/KAT3B activity in the mouse brain impairs memory consolidation (Viosca et al. 2010; Oliveira et al. 2007, 2011; Lipinski et al. 2019). Moreover, CBP/KAT3A and P300/KAT3B are genetically linked to Rubinstein-

Taybi syndrome, an autosomal dominantly inherited form of mental retardation that affects 1 in 125,000 individuals (Petrij et al. 1995; Oike et al. 1999). It is therefore important to note that administration of an HDAC inhibitor was able to ameliorate the phenotypes in KAT3A/CBP mutant mice (Alarcon et al. 2004). KAT3A/CBP has also been linked to Alzheimer's disease. For example, it was found that phospho-CBP levels are downregulated in the 3xAD mice and that viral-mediated overexpression of CBP was able to ameliorate deficits in phospho-CBP levels and memory function (Caccamo et al. 2010, CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease). Moreover, deletion of presenilins 1 and 2 in mice affected CBP-mediated gene expression (Saura et al. 2004). There is also evidence PCAF/KAT2B is required for memory formation (Maurice et al. 2008; Duclot et al. 2010; Wei et al. 2012; Mitchnick et al. 2016). PCAF has also been linked to AD pathogenesis. One study found that mice which lack PCAF (KAT2*B*) are resistant to the detrimental effects of amyloid beta peptides that are directly injected into the lateral ventricles of mice (Duclot et al. 2010). This data is somewhat in conflict with the fact that the same PCAF mutant mice were found to show impaired memory function (Maurice et al. 2008), and it remains to be seen if reducing PCAF levels would have similar effects in more established models for amyloid pathology. Nevertheless, such findings remind us that the role of histone and protein acetylation in AD is likely very complex and should not be exclusively reduced to the hypothesis that inhibition of HDACs and activation of HATs would ameliorate cognitive impairment.

Studies from our laboratory used an unbiased RNA-sequencing approach to compare the expression of the 18 HATs in the hippocampal CA1 region of adult mice, a region that is intimately linked to memory formation (Stilling et al. 2014) (Table 1). These data revealed that GCN5/KAT2A and TIP60/KAT5 are the highest expressed HATs in this brain region, at least at the mRNA level. Consequently, deletion of GCN5/KAT2A from excitatory forebrain neurons of the adult brain leads to severely impaired spatial reference and associative memory consolidation, as well as impaired hippocampal long-term potentiation (LTP), that is considered to be a molecular correlate of memory formation (Stilling et al. 2014). Loss of GCN5/KAT2A also had a profound impact on hippocampal gene expression that was linked to the acetylation of histone 4 at lysine 12 and histone 3 at lysine 14 and 18 as well as the acetylation of the p65, a co-regulation of the transcription factor NFkappaB (Stilling et al. 2014). Similarly, unpublished data from our laboratory suggest that deletion of the other highly expressed HAT in the hippocampus, namely, KAT5/TIP60, leads to severe memory impairment and a massive deregulation of neuronal gene expression when deleted from excitatory forebrain neurons. In line with these data, pharmacological inhibition of KAT5A/TIP60 impaired remote memory retrieval, a finding that was linked to the role of KAT5A/TIP60 in the acetylation of the histone variant H2A.Z (Narkaj et al. 2018). KAT5A7TIP60 has also been implicated with Alzheimer's disease. To this end it was shown that increased TIP60 activity can rescue amyloid beta-induced neurotoxicity (Pirooznia et al. 2012) and axonal transport deficits in a *Drosophila* model (Johnson et al. 2013). In line with the data that KMTs do not exclusively act on histones, loss of

TIP60 activity in drosophila causes reduced microtubule acetylation (Sarthi and Elefant 2011). Nevertheless, the protective effect of increasing TIP60 activity is – at least in part – also linked to the expression of genes that regulate apoptotic cell death (Pirooznia et al. 2012) and axonal transport (Johnson et al. 2013). A potential role for TIP60 in Alzheimer's disease has been furthermore suggested even earlier since it was found that TIP60 regulates gene expression in a complex with the amyloid precursor protein intracellular domain (AICD) (Cao et al. 2004; Müller et al. 2013). Moreover TIP60 plays a critical role in orchestrating the cellular response to DNA damage (Kaidi and Jackson 2013) which is emerging as another critical player in AD pathogenesis (Mao and Reddy 2011; Herrup et al. 2013).

Comparatively little is known about the role of the other HATs in the adult brain and memory function. HAT1/KAT1 expression increases in mice treated with antidepressants, but the functional consequences are not understood (Kocki et al. 2018). These data are however in line with the observation that neuronal histone modifications are altered in depression (Sun et al. 2013). Myst4/KAT6B has (Merson et al. 2006) been linked to adult neurogenesis, a process that has been linked to cognitive function (Ernst and Frisén 2015), while MYST1/KAT8 has been linked to Alzheimer's disease by a recent GWAS study (Marioni et al. 2018). RNAi-mediated knockdown of NCOA1/KAT13A in the hippocampal CA1 region of mice impairs spatial reference memory and LTP which was accompanied by reduced synapse density (Bian et al. 2018). The other HATs have not been linked to memory function yet, although mutations in TAF1/KAT4 are linked to X-linked dystonia-parkinsonism (Bragg et al. 2019). It is important to mention that HATs act as part of larger protein complexes and proteins with HAT activity are also part of the RNA-pol II complex (Lee and Workman 2007). This is of particular interest since acetyl-CoA is the main donor of acetyl groups essential for histone acetylation. It was recently shown that acetyl-CoA synthase 2 (ACSS2) is the main enzyme that provides acetyl-CoA to histone acetylation in the adult brain and that reduced ACSS2 levels lead to memory impairment (Mews et al. 2017). This is in line with previous data suggesting that age-associated memory decline is linked to reduced hippocampal citrate levels (Peleg et al. 2010), a main donor for acetyl-CoA synthesis.

The activity of HATs is counteracted by the histone deacetylases (HDAC) that are grouped into the zinc-dependent HDACs and the non-zinc-dependent sirtuins. A number of excellent reviews discuss the role of sirtuins and HDACs in various cellular systems (Haigis and Sinclair 2010; Donmez 2013; Fischer et al. 2010; Fischer 2014b). Here we will specifically focus on the role of the zinc-dependent HDACs in memory function. In line with the data that have generally shown that loss of HAT activity in linked to memory impairment, it was found that pharmacological inhibition of HDACs enhances the consolidation of memories in rodents (Levenson et al. 2004; Fischer et al. 2007; Stefanko et al. 2009; Federman et al. 2009; Benito et al. 2015; Agís-Balboa et al. 2017).

The 11 mammalian zinc-dependent HDACs belong to an ancient protein family and require a Zn^{2+} ion as cofactor (Gregoretti et al. 2004; de Ruijter et al. 2003). Under naïve conditions, all HDAC genes are expressed within the adult rodent brain

(Broide et al. 2007). Mice that lack HDAC1 or overexpress it in all neurons from early developmental stages show no impairments in contextual fear learning or spatial memory formation (Guan et al. 2009), suggesting that HDAC1 has no obvious role at least in the abovementioned types of memory function. HDAC1 was however found to be essential for fear extinction learning, a process that is important for the treatment of neuropsychiatric diseases associated with aversive behaviors as they occur in post-traumatic stress disorder (Bahari-Javan et al. 2012). Here, the induction of immediate early genes was suppressed via HDAC1-mediated deacetylation of H3K9 and subsequent H3K9 tri-methylation (Bahari-Javan et al. 2012). Although HDAC1 and HDAC2 are close homologues that derived from gene duplications, their roles in memory function differ. Overexpression of HDAC2 in neurons impaired contextual fear learning and spatial memory formation in mice, while deletion of HDAC2 in neurons from early developmental stages improved memory function and synaptic plasticity (Guan et al. 2009). Notably, enhanced learning behavior in HDAC2 knockout mice correlated with increased hippocampal H4K12 acetylation, while H3K14 acetylation was not affected. Although the authors measured bulk changes, these data are interesting taking into account that genome-wide analysis of chromatin in the aging hippocampus suggested a key role for H4K12 acetylation in age-associated memory impairment (Peleg et al. 2010). Later studies suggest that the memory enhancing effect of HDAC2 reduction is linked to the activity of the transcription factor SP1 (Yamakawa et al. 2017). On the structural level, loss of HDAC2 increased the number of synapses (Guan et al. 2009) which is in line with a role of HDAC2 – but also HDAC1 – in synapse formation during development (Akhtar et al. 2009). Loss of HDAC2 was also found to improve fear extinction learning (Morris et al. 2013) which is opposite to the function of HDAC1 (Bahari-Javan et al. 2012). HDAC2 was found to bind promoter regions of genes linked to memory formation, but the precise mechanisms by which HDAC2 acts as a memory repressor are not well understood (Guan et al. 2009). One study showed that HDAC2 is essential for the survival of adult born neurons in the dentate gyrus (Jawerka et al. 2010). Since adult neurogenesis has been linked to memory function, it is clear that the role of HDAC2 in the adult brain awaits a more detailed analysis. An important next step would be to understand the role of HDAC1 and 2 in a cell type-specific manner. A recent study suggests that HDAC2 is produced in many neuronal cell types and in oligodendrocytes but not in astro- or microglia (Yao et al. 2013), a view that is however not undisputed since recent data point to an important role of HDAC1 and 2 in glia cells (Wendeln et al. 2018). HDAC1 has been also linked to neuropsychiatric diseases (Jakovcevski et al. 2013a). For example, a number of studies have demonstrated a role for HDAC1 in schizophrenia. Thus, HDAC1 was upregulated in postmortem brain tissue of schizophrenia patients (Dong et al. 2007; Sharma et al. 2008; Bahari-Javan et al. 2017). In a recent study, our laboratory was able to show that HDAC1 is increased specifically in schizophrenia patients that encountered early life stress (Bahari-Javan et al. 2017). Interestingly, this upregulation of HDAC1 was linked to DNA methylation-dependent regulation of the HPA axis and was also prominent in corresponding human blood samples (Bahari-Javan et al. 2017), suggesting the possibility that HDAC1 might

serve as a biomarker for stratified therapy of patients with an unfavorable course of the disease (Bahari-Javan et al. 2017). Similar to the data available for HDAC2, mice that lack HDAC3 in the adult hippocampus show enhanced memory function (McQuown et al. 2011) which is mechanistically linked to the regulation of the Nr4a gene (Kwapis et al. 2019). The function of HDAC8 in adult brain has not been addressed in detail, but a recent study found that an HDAC inhibitor with some selectivity toward HDAC8 improves memory function in rats (Yang et al. 2013). In conclusion, it appears that the class I HDACs act as molecular inhibitors of memory formation and are thus potential therapeutic targets to ameliorate memory impairment.

As for the class II HDACs (Table 1), HDAC4 is known to shuttle between the cytoplasm and the nucleus of cultured hippocampal neurons in response to calcium signaling and CamKII activity (Chawla et al. 2003; Backs et al. 2006). In a *C. elegans* model, deletion of HDAC4 gene increases long-term memory for thermosensation in a CamKII-dependent manner (Wang et al. 2011). Specific expression of mammalian HDAC4 in the nucleus was able to revert this phenotype suggesting that nuclear export of HDAC4 is a critical process for memory formation. In line with this data, cytoplasmatic expression of HDAC4 increased memory formation in wild-type worms (Wang et al. 2011), suggesting that during learning HDAC4 regulates counteracting molecular processes in the nucleus and the cytoplasm. In line with these data, another study demonstrated that overexpression of HDAC4 impairs long-term memory in a *Drosophila* model (Fitzsimons et al. 2013). In contrast to such findings, a recent study suggests that HDAC4 is essential for memory function in mammalian systems. Mice that lack HDAC4 in the adult forebrain exhibit impaired hippocampus-dependent memory formation and plasticity (Kim et al. 2012b). This data is in line with findings showing that haploinsufficiency of HDAC4 is linked to mental retardation in humans (Williams et al. 2010). Another study confirmed that lack of HDAC4 in the adult brain results in impaired memory function and synaptic plasticity in mice and could identify synaptic plasticity genes that are regulated by nuclear HDAC4 (Sando et al. 2012). There is also evidence that HDAC4 controls genes essential for adult neurogenesis (Saha et al. 2019) that contribute to the development of psychiatric phenotypes in response to stressful events (Maddox et al. 2018).

Regarding HDAC5, there is a substantial amount of evidence implicating this HDAC with the mechanisms linked to drug abuse (Smith and Kenny 2018). Loss of HDAC5 in the nucleus accumbens renders mice hypersensitive to chronic cocaine (Renthal et al. 2007) and regulates cocaine-conditioned behaviors (Taniguchi et al. 2017), while 2-month-old mice that lack HDAC5 from the adult forebrain show no changes in hippocampus-dependent memory formation (Kim et al. 2012b). A role of HDAC5 in memory formation may not only be brain-region but also age-related. To this end 10-month-old mice that lack HDAC5 show hippocampus-dependent memory disturbances (Agis-Balboa et al. 2013).

HDAC6 is best known for its acetylation of tubulin or heat shock protein 90 (Govindarajan et al. 2013). Loss of HDAC6 has mild impact on memory function in wild-type mice but improves cognition in mouse models for Alzheimer's disease-related protein aggregation (Govindarajan et al. 2013; Fan et al. 2018; Selenica et al.

2014) or Fragile X syndrome (Kozikowski et al. 2019). HDAC7 was found to be downregulated in response to memory training in mice, which was linked to the expression of genes essential for long-term memory consolidation (Jing et al. 2017). Little is known on the role of HDAc9 in the adult brain, but a recent study points to a role of an HDAC9-dependent circRNA in dementia (Lu et al. 2019). Moreover, HDAC9 has been linked to dendritic plasticity (Sugo et al. 2010) and is associated with stroke and schizophrenia (Lang et al. 2011; Markus et al. 2013). Almost nothing is known about the role of HDAC10 and 11 in the adult brain. In conclusion, several HATs and HDACs have emerged as key players in memory function. Future research will be essential to understand how these epigenetic enzymes are regulated and which gene expression pathways they control. Moreover, it is important to mention that this enzymatic machinery not only regulates the acetylation state of specific histone proteins but also affects non-histone proteins such as transcription factors but also proteins generally linked, for example, to metabolic processes (Choudhary et al. 2009).

1.3 Histone Methylation: H3K4

In addition to acetylation, another well-studied histone modification is methylation (Vaquero et al. 2003). Similar to histone acetylation, it is regulated by the counteracting activity of histone methyltransferases (HMTs) and histone demethylases (HMDs). However, in contrast to acetylation, the lysine residues of histones can be either mono-, di-, or tri-methylated which is catalyzed by specific enzymes. The functional consequences of histone methylation are thus also more complex and have been either linked to active euchromatin or the formation of facultative and constitutive heterochromatin that is associated with gene silencing. As such, there are more HMTs and HDMs than there are HATs and HDACs, and their general role has been discussed in a number of review articles (Shi 2007; Shi and Whetstine 2007; Badeaux and Shi 2013). In the context of memory formation, most data has been generated on the role of histone 3 methylation (H3K4me3) that is enriched around transcription start site (TSS) regions of actively transcribed and/or poised genes (Guenther et al. 2006), whereas histone 3 lysine 4 monomethylation (H3K4me1) is enriched at enhancers (Heintzman et al. 2009). H3K4 methylation is mediated by SET proteins. Set1 is the only H3K4 methylase in yeast (Roguev et al. 2001), whereas in *Drosophila*, three different SET proteins – Set1, trithorax (trx), and trithorax-related (trr) – are expressed (Mohan et al. 2011). Mammals, in turn, possess six SET-related H3K4 methyltransferases – Kmt2a (Mll1), Kmt2b (Mll2), Kmt2c (Mll3), Kmt2d (Mll4), Setd1a, and Setd1b – that have been linked to mono-, di-, and tri-methylation. These proteins are subdivided into three groups, based on their homology to each other and to the corresponding *Drosophila* homologues. Kmt2a/Kmt2b are related to trx, Kmt2c/Kmt2d to trr, and Setd1a/Setd1b to Set1 (Shilatifard 2012). The existence of several homologous histone-modifying enzymes mediating the same modifications raises the question of redundancy vs. specificity of

their actions. This is especially true for brain regions such as the hippocampus, which is intimately linked to memory function and cognitive diseases, where all six H3K4-KMTs are strongly expressed (Kerimoglu et al. 2017}. It is also important to note that mutations in any of the six H3K4-KMTs are linked to various rare diseases that include cognitive phenotypes and intellectual disability (Kleefstra et al. 2014).

In line with this, a number of studies implicated the regulation of H3K4me3 with memory function. For example, it was found that H3K4me3 correlates with the expression of glutamate receptors in the human brain (Stadler et al. 2005). Moreover, bulk levels of hippocampal H3K4me3 were found to increase in response to fear learning (Gupta et al. 2010), and mice that constitutively lack one allele of the H3K4-KMT KMT2A display impaired memory formation (Gupta et al. 2010). In line with this, conditional deletion of KMT2A from neurons of the adult brain impaired synaptic plasticity and working memory (Jakovcevski et al. 2015). Another study also demonstrated a role for the H3K4-specific HMT Mll2/*KMT2B* in memory function. Mice that lack KMT2B in the dorsal dentate gyrus of hippocampal region show memory impairment that is linked to deregulation of learning-relevant genes (Kerimoglu et al. 2013). Loss of KMT2B not only affected H3K4me3 at the promoter regions of learning-regulated genes but also reduced H3K9 acetylation, while H4K16 acetylation and H3K4me1 were unaffected at the same gene promoters (Kerimoglu et al. 2013). Such data further confirm the view that histone methylation and histone acetylation are tightly linked and also demonstrate the need to better understand the protein complexes that regulate chromatin plasticity in the adult brain. Especially the latter is in utmost importance since recent data compared the role of two closely related H3K4me3 KMTs – KMT2A and KMT2B – in excitatory neurons of the adult brain (Kerimoglu et al. 2017). These data show that loss of either enzyme leads to the impairment of hippocampus-dependent memories, but it was somewhat surprising to see that the gene expression pathways controlled by KMT2A and KMT2B in the hippocampus were entirely different. In fact, loss of neuronal KTM2A mainly affected gene expression pathways linked to neuronal identity and synaptic plasticity, while loss of KMT2B was linked to more general metabolic functions (Kerimoglu et al. 2013, 2017). Preliminary data from our lab suggest that similar observations are made for other KMTs that regulate H3K4me3. Mechanistically this phenomenon is presently not understood, but multiple explanations can be considered. Different KMTs could simply occupy different genomic regions, a hypothesis that is at present difficult to be tested since antibodies that allow the reliable analysis of KMTs in ChIP-seq experiments are necessary for this approach. Some evidence for this hypothesis stems however from the finding that in the neurons of KMT2A mutant mice, the deregulated genes contain different transcription factor binding sites when compared to KMT2B (Kerimoglu et al. 2017). KMTs may however also occupy similar genomic regions but might be regulated via different posttranslational modifications that are associated with distinct protein complexes. This could have different function outcomes. For example, KMT2A has been also linked to the regulation of higher-order chromatin structure and long-distance interactions (Bharadwaj et al. 2014). Mechanistic data on the other H3K4 HMTs in memory formation are comparatively rare. Loss of KMT2D in mice leads

to phenotypes that partially recapitulate the Kabuki syndrome including hippocampus-dependent memory impairment, which can be rescued by the inhibition of HDAC inhibitors (Bjornsson et al. 2014) or a ketogenic diet (Benjamin et al. 2017). These data are highly interesting, since they suggest that defects in neural H3K4me3 can be attenuated by HDAC inhibitors that increase histone acetylation. While this is in line with other finding suggesting that also in neurons H3K4me3 is functionally coupled to histone acetylation (Kerimoglu et al. 2013, 2017), data from T-cells had suggested that genes which do not carry H3K4me3 cannot be regulated by HDAC inhibitors (Wang et al. 2009). Virtually nothing is known on the role of SETD1A in the adult brain, but it has been genetically linked to autism and schizophrenia (Singh et al. 2016; Takata et al. 2016). Also on the role of SET1B in the adult brain, there is so far little data available. Mutations in the corresponding genes are also linked to intellectual disability and autism (Labonne et al. 2016; Hiraide et al. 2018).

Four histone demethylases (KDMs) counteract the activity of the H3K4-KMTs. These are KDM5A (JARID1A), KDM5B (JARID1B), KDM5C (JARIC1C), and KDM5D (JARID1C). Little is known on the role of KDM5A and KDM5D. There is in vitro evidence that reducing the levels of KDM5B might increase neurogenesis in the subventricular zone of the adult brain (Zhou et al. 2016). Mutations in KDM5C have been linked to mental retardation (Jensen et al. 2005; Rujirabanjerd et al. 2012) and to short-term memory deficits in female humans (Simensen et al. 2012). A recent study analyzed mutant mice that lack one KDM5C allele either constitutively from early developmental stages and in addition tested mice that lack KDM5C in excitatory neurons of the adult brain (Scandaglia et al. 2017). While loss of KMD5C from developmental stages leads to memory impairment, this effect is less pronounced when KDM5C is deleted at adult stages. Similar findings are observed when H3K4me3-dependent gene expression was analyzed. The finding that deletion of KDM5C from the adult brain has no severe phenotype is interesting taking into account that reduced H3K4me3 has been linked to adult onset memory impairment and cognitive disease such as Alzheimer's disease. These data suggest that inhibition of KMD5 proteins may ameliorate corresponding disease phenotypes. In line with this, a study available via bioRxiv shows that the phenotypes including memory impairment which are linked to the loss of KMT2A are ameliorated when these mutant mice are crossed with mice that lack KDM5C (Vallianatos et al. 2019). In conclusion, the current data point to a key role of H3K4 methylation in cognitive function and suggest that loss of H3K4 methylation in neurons is linked to memory impairment. In turn, drugs that increase H3K4 methylation could be suitable avenues for the treatment of cognitive diseases. Whether this could be achieved by the inhibition of HDAC or KDMs or both needs to be investigated. Also, clearly more research on the role of the H3K4 regulating enzymes in brain circuitries linked to memory function is needed.

1.4 Histone Methylation: H3K9

In addition to H3K4 methylation, there is a substantial amount of data on H3K9 methylation in the adult brain that is a mark for heterochromatin-mediating gene silencing. Similar to the situation described for H3K4 methylation, a number of different enzymes that are all expressed in the adult brain mediate H3K9 methylation. These are KMT1A (SUV39H), KMT1B (SUV39H2), KMT1C (G9A/EHMT2), KMT1D (GLP/EHMT1), KMT1E (SETDB1), and KMT8A (PRDM2).

While little is known on the role of H3K9 tri-methylase KMT1A in the adult brain, KMT1B was found to be upregulated in the hippocampus of rats in response to restraint stress, a procedure that affects memory function (Hunter et al. 2012). KMT1C and KMT1D have been linked to H3K9 mono- and di-methylation and act mainly as a complex. Pharmacological compounds affect in most cases both proteins. KMT1C and KMT1D were linked to intellectual disability and autism spectrum disorder (Koemans et al. 2018). Mice that lack KMT1C in the adult forebrain develop mental retardation-like phenotypes (Schaefer et al. 2009). However, loss of KMT1C in the nucleus accumbens was shown to increase cocaine-induced neuronal plasticity (Maze et al. 2010). In *Drosophila*, loss of KMT1C impaired memory formation (Kramer et al. 2011). Another study found that pharmacological inhibition of KMT1C/KMT1D in the entorhinal cortex facilitated memory function, while administration of the same inhibitor into the hippocampus resulted in impaired memory function in mice (Gupta-Agarwal et al. 2012). Another KMT1C/KMT1D inhibitor was found to enhance hippocampal LTP (Sharma et al. 2018), while yet another compound reduced anxiety when administered to adult animals, whereas administration during development increased anxiety when the animals were adult (Wang et al. 2018). Another recent study reported that pharmacological inhibition of KMT1C/KMT1D rescues memory impairment and synaptic function in a mouse model for amyloid deposition (Zheng et al. 2019). In sum, these data do not leave a clear picture on the role of KMT1C/KMT1D in the adult brain, but they clearly demonstrate an important role for these enzymes. One likely explanation to this could be the different roles of KMT1C/KMT1D in the developing and adult brain. Thus, timing of KMT1C/KMT1D manipulation appears to be essential for the interpretation of the resulting phenotypes.

Increased expression of KMT1E in the forebrain of mice reduced depressive-like behavior via a mechanism that involved regulation of NMDA receptor subunit 2B (Jiang et al. 2010). KMT1E is linked to H3K9-tri-methylation and was also found to be an essential regulator chromosomal conformation in mouse neurons of the adult brain (Bharadwaj et al. 2014; Jiang et al. 2017). KMT8A has been linked to mono-methylation of H3K9. It has been implicated with the detrimental effect of alcohol abuse (Barbier et al. 2017).

H3K9 methylation is reversed by the action of H3K9 KDMs that are however also known to affect other methylation sites such as H3K36. These are KDM2A (JHDM2A), KDM4A (JMJD2A), KDM4B (JMJD2B), KDM4C (JMJD2C),

KDM4D (JMJD2D), and KDM4E (JMJD2E). KDM4A was found in a complex with HDAC1 and protein phosphatase 1 (PP1) which was required for memory formation (Koshibu et al. 2009). When KMT4B was deleted from adult forebrain neurons, the authors observed altered morphology of dendritic spines. Moreover mutant mice exhibited impaired working memory (Fujiwara et al. 2016). Another KMD that acts on H3K4 and K3K9 is KMD1 (LSD1). It was shown that pharmacological inhibition of LSD1 impairs memory function in mice (Neelamegam et al. 2012). In line with this data, recent data show that deletion of KDM1 from adult forebrain neurons in mice leads to neurodegeneration and memory impairment and induces gene expression changes linked to Alzheimer's disease and frontotemporal dementia (Christopher et al. 2017).

In conclusion, the current data suggest that H3K4 methylation, as well as the activity of the corresponding HMTs and HDMs play a role in memory formation, are heavily linked to intellectual disability disorders and that targeting this machinery could a suitable approach for the treatment of cognitive diseases.

1.5 Histone Methylation: H3K27

Tri-methylation of H3K27 has been linked to facultative heterochromatin and gene silencing, while its mono- and di-methylation has been linked to active gene expression. H3K27me3 counteracts the role of H3K4me3, and both marks are often present at the same gene promoter which is then called bivalent promoter since they carry an active and repressive mark. Such genes are often induced by specific stimuli; hence they are repressed but can be induced rapidly by decreasing, for example, H3K27me3. Thus, H3K27me3 is associated with facultative heterochromatin. Interestingly, large portions of non-facultative heterochromatin are also marked by histone H3K9me3 and are thereby tethered to the nuclear lamina and associate with it in what is called lamina-associated domains (LADs) (van Steensel and Belmont 2017). Interestingly a recent publication elegantly showed that mechanical forces derived from tensile loading in human epithelial progenitor cells resulted in a switch from the constitutive heterochromatin marker H3K9me3 to the facultative H3K27me3 (Le et al. 2016). Deregulation of H3K27 methylation has been linked to neurodegenerative diseases. For example, changes in H3K27me3 a specific form of frontotemporal dementia is caused by mutations in the C9ORF72 genes that result in GGGGCC expanded repeats. It was found that mutant H3K27me3 level is increased in the mutant C9ORF72 gene and contributes to its downregulation (Belzil et al. 2013). Mutant C9ORF72 variants are also linked to translation leading to the generation of dipeptide repeat proteins (DRPs). A recent study showed that one of these DRPs, namely, proline-arginine (PR)-DRP, causes heterochromatin abnormalities linked to altered H3K27 methylation (Zheng et al. 2019). H3K27 methylation is mediated by the polycomb repression complex 2 (PRC2) with the essential subunits KMT6A (EZH2) and KMT6B (EZH1). KMT3F methylates H3K4 and H3K27. Neuronal deletion of KMT6A in mice

from early developmental stages resulted in hippocampal memory impairment and reduced adult neurogenesis (Zhang et al. 2014). Another study found that in response to memory training, KMT6A regulated H3K27me3-dependent expression of the PTEN gene (Jarome et al. 2018). Ataxia-telangiectasia (A-T), also named Louis-Bar syndrome, is a rare progressive neurodegenerative disease that leads to cerebellar ataxia and caused by mutations of the ATM kinase. ATM was found to act on KMT6A, and the corresponding changes in H3K27 methylation have been linked to the disease progression (Li et al. 2013). Regarding KMT6B, it was shown that its expression is regulated by microRNA-132 (Johnstone et al. 2018), a microRNA intimately linked to memory formation (Fischer 2014a). The same study reports the microRNA-132-dependent downregulation of KMT6B in mice that were chronically exposed to antipsychotics (Johnstone et al. 2018). Consequently, knockdown of KMT6B in the prefrontal cortex affected motivational behavior. Another H3K27 KMT is KMT3F (NSD3) that can however also methylate H3K4. Nothing is known on the role of this enzyme in the adult brain. Among the H3K27 KDMs, KMD6A (UTX) and KDM6B (JMJD3) are rather specific to the demethylation of H3K27me2 and HeK27me3. KMD6A is encoded on the X chromosome, and there is indeed evidence for a sex-specific expression of KMD6A in neurons; more specifically KMD6A levels are higher in females (Xu et al. 2008). KMD6A has also been linked to the Kabuki syndrome, and deleting KDM6A from neurons from early developmental stages in mice results in impaired hippocampus-dependent memory formation and reduced LTP (Tang et al. 2017). Gene expression analysis revealed that KDM6A control genes linked to serotonergic signaling (Tang et al. 2017). Knockdown of KDM6B in hippocampal neurons impaired the stimulus-dependent expression of candidate genes (Wijayatunge et al. 2014). There is also evidence that KMD6B function is essential to keep neurogenesis genes in a poised state and thereby regulates neurogenesis in the developing and neurogenic niches of the adult brain (Park et al. 2014). Moreover, pharmacological inhibition of KMD6A in mice was shown to impair reinstatement of cocaine reward memory (Zhang et al. 2018). Nothing is known on the role of KDM7B on memory function in the adult brain.

In sum, although comparatively less studied than H3K4, the current data link H3K27 methylation and the corresponding enzymatic machinery to cognitive function and brain diseases.

1.6 Other Histone Methylation Sites

Little is known about the dynamic regulation of H3K36 methylation, a mark linked to complex regulation of gene expression depending on its mono-, di-, or tri-methylation, in the adult brain. This histone lysine residue can be methylated by KMT3A (SET2) that mediates H3K36me3, while KMT3B (NDS1) and KMT3C (SMYD2) mainly regulate H3K36 mono- and di-methylation. H3K36 demethylation is catalyzed by KDM2A (JHDM1A), KDM2B (JHDM1B), and KDM8 (JMJD5) that act on H3K36 mono- and di-methylation. Besides the observation that KDM2B

has been genetically linked to intellectual disability and neuronal progenitor dysfunction leading to exencephaly (Fukuda et al. 2011; Labonne et al. 2016), nothing is known on the role of these enzymes in the adult brain. Demethylation of H3K36me3 is mediated by KDM4A–E that have been discussed above. Another histone modification is H3K79 methylation which has been linked to active gene expression but is also implicated with more complex regulations depending on the cellular context. Dynamic changes in H3K79 methylation are involved with neurodevelopmental processes (Büttner et al. 2010) and are reduced in FTLD/ALS patients that carry mutations in the C9ORF72 gene (Belzil et al. 2013). H3K79 mono-, di-, and tri-methylation is mediated by KMT4 (Dot1) that play a role in cortical development (Roidl et al. 2016). Demethylation of H3K79 has been linked to the activity of KDM2B that also acts on H3K36 and has been discussed above. H4K20 is so far the main methylation site on histone 4. H4K20me1 has been linked to active transcription, while H4K20me2 occurs during specific cellular processes such as cell cycle control. In contrast H4K20me3 is linked to repression of gene expression. Reduced H4K20me3 was observed in FTLD/ALS patients with the C9ORF72 mutation (Belzil et al. 2013) and in a mouse model for accelerated aging (Wang et al. 2010). H4K20 methylation is mediated by KMT5A (SET8) that mediated mono-methylation, while KMT5B (SUV4-20 h1) and KMT5C (SUV4-20H2) control di- and tri-methylation. While nothing is known on the role of KMT5A and C in the brain, KMT5B is associated with autism and developmental-disability biases (Stessman et al. 2017). A very interesting finding was that LSD1n is a H4K20 demethylase. LSD1n is a neuronal-specific splice variant of KMT1 (LSD1). Interestingly, while LSD1 affect H3K4 and H3K9, LSD1n de-methylates H4K20. Genetic ablation of LSD1n caused memory impairment and deregulation of neuronal gene expression (Wang et al. 2015).

1.7 Other Histone Modifications

Histones can also be reversibly ubiquitylated, which is probably the most severe form of a histone modification considering that a 76-amino-acid polypeptide is added to a histone tail. Histone ubiquitylation occurs mainly at lysine 119 of H2A (H2AK119ub) and lysine 120 of histone H2B (H2BK120ub). Both modifications occur as mono-ubiquitylation are thus not priming histones for degradation but serve signaling function that are diverse and have been linked to activation and repression of gene expression depending on the cellular context (Allis et al. 2007b). Early studies already observed increased H2A and H2B ubiquitylation in the aging mouse brain (Morimoto et al. 1993) which is in line with more recent data showing that elevated H2A ubiquitylation is a general marker for aging in a *Drosophila* model which is conserved in rodents, primates, and humans and that reduction of H2AK119ub in *Drosophila* increases life span (Yang et al. 2019). H2AK119ub not only increases in aging but also in neurodegenerative disease such as Huntington chorea (McFarland et al. 2013). Ubiquitination of histones is, for example, mediated by the E3 ubiquitin ligases RNF20 (BRE1A) and RNF40 (BRE1B) which have been

linked to astrocyte differentiation (Liang et al. 2018). Histones can also be phosphorylated, and a prime example is the phosphorylation of the noncanonical histone gamma-H2AX that plays an important role in the DNA-damage response (Kinner et al. 2008). Interestingly, activity-induced DNA damage has been implicated with synaptic plasticity, memory consolidation, and neurodegenerative disease (Suberbielle et al. 2013; Madabhushi et al. 2015). Many other histone modifications have been reported (Vaquero et al. 2003), but their role in the adult brain remains to be studied. It has also been mentioned that in addition to the comparatively well-studied acetylation and methylation modifications of histone 3 and 4, proteome analysis suggests that more sites may exist, which still await to be studied in the brain (Brunner et al. 2012).

In addition to histone modifications, it is interesting to mention that under certain conditions, the canonical histones can be replaced by histone variants. A prime example is the replacement of H2A with H2A.Z that is considered represent a mechanisms for a transcriptional memory (Buschbeck and Hake 2017). Other variants are, for example, H3.3 or macroH2A. While in the brain H3 is constantly replaced by H3.3 along aging, so that older individuals contain almost exclusively to the replication-independent H3.3, the amount of H2A.Z was initially found to be rather constant (Piña and Suau 1987). Interestingly, it was shown that memory training in mice induced the eviction of H2A.Z at selected genes in the hippocampus and cortex of mice. Moreover H2A.Z levels were found to increase in the hippocampus of aging mice, and viral-mediated knockdown of H2A.Z improved memory function in mice (Zovkic et al. 2013; Stefanelli et al. 2018). Interestingly KAT5/TIP60 has been implicated with the deposition of H2A.Z, and more recent data suggest that inhibition of this process can improve memory consolidation in mice (Narkaj et al. 2018). While H3.3 has been linked to the general aging process (Bano et al. 2017), little is known on it and other histone variants in the adult brain, and also the role of reader proteins is only beginning to emerge.

2 Conclusion

Histone modifications are dynamically regulated during memory formation and in cognitive diseases. Histone acetylation appears to be a prerequisite for neuronal plasticity, and loss of the corresponding KMTs leads to memory impairments. In turn, the inhibition of class I HDAC has emerged as a suitable and promising approach to improve memory function in cognitive diseases. Histone methylation is also critical for proper neuronal plasticity, but the picture is more complex. Mutations in many of the enzymes machinery that control the various histone methylation events are linked to intellectual disability disorders. Best studied is the role of H3K4 and H3K9. Future research is needed to explore the full spectrum of histone modifications in the adult brain and understand how these modifications build a combinatorial code that controls transcriptome plasticity in neuronal circuitries.

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Disease Modeling of Neuropsychiatric Brain Disorders Using Human Stem Cell-Based Neural Models

Johanna Kaindl and Beate Winner

Contents

Abstract Human pluripotent stem (PS) cells are a relevant platform to model human-specific neurological disorders. In this chapter, we focus on human stem cell models for neuropsychiatric disorders including induced pluripotent stem (iPS) cell-derived neural precursor cells (NPCs), neurons and cerebral organoids. We discuss crucial steps for planning human disease modeling experiments. We introduce the different strategies of human disease modeling including transdifferentiation, human embryonic stem (ES) cell-based models, iPS cell-based models and genome editing options. Analysis of disease-relevant phenotypes is discussed. In more detail, we provide exemplary insight into modeling of the neurodevelopmental defects in autism spectrum disorder (ASD) and the process of neurodegeneration in Alzheimer's disease (AD). Besides monogenic diseases, iPS cell-derived models also generated data from idiopathic and sporadic cases.

Keywords Disease modeling · hiPSCs · hiPSC-derived neurons · Neuropsychiatric disorders · Organoids

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1 Introduction

Neuropsychiatric disorders are associated with heavy social and economic burden, contributing to the leading cause of disabilities worldwide (Whiteford et al. 2017; WHO 2014). Psychiatric diseases, such as autism spectrum disorder (ASD), schizophrenia (SCZ), and bipolar disorder (BPD), have heterogeneous etiologies. Symptoms are variable and complex, including abnormal social interaction and communication, restricted behavior, and deficits in cognition, combined with the lack of objective biomarkers. This heterogeneity has been challenging for experimental studies (Soliman et al. 2017). Genetically engineered mice frequently carry single mutations in relevant genes. The limitation of these models is that they only recapitulate certain aspects of the human disease phenotypes. However, due to the differences in brain size and architecture and development of the rodent and the complex human brain, studying human-specific neurological disorders in other species has certain limits. Therefore, a wide range of studies considering the pathology of neuropsychiatric disorders have been performed using brain imaging, genomic studies, and analysis of postmortem tissue. Genome-wide association studies (GWAS) reported heterogeneous combinations of numerous alleles of small effect, besides the rare and highly penetrant mutations (Gratten et al. 2014). However, the identification of underlying mechanisms and pathways beyond the genetic risk associations in cellular disease models has been challenging. Since many neuropsychiatric diseases appear sporadic, affect complex high-order brain functions, and involve multiple cell types across different brain regions, new human-specific disease models are needed for preclinical studies and understanding of disease-causing phenotypes. Human stem cell-based models for neuropsychiatric disorders have continuously emerged over the last decade. Here we review the recent progress of disease modeling of neuropsychiatric disorders using human stem cell-based models. Since over 500 studies covering human pluripotent stem cells and neurosciences have been published over the last 10 years, our review comprises a selection of two disease entities and exemplary studies on neuropsychiatric disease modeling.

2 Disease Modeling of Neuropsychiatric Brain Disorders Using Human Stem Cell-Based Neural Models

The first and crucial step when planning human disease modeling for neuropsychiatric diseases is to decide for the specific cell type/types needed for the experiment. This decision tree includes various experimental levels: (1) starting material, (2) necessary steps of reprogramming or genome editing, (3) the technique of generation of neural progenitors and neurons, and (4) neurotransmitter phenotypes. Depending on the hypothesis of the experiment, these are the first crucial decisions to be made. Therefore, within this chapter, we will describe the basic principles to generate neural models and neurons and discuss respective selection criteria.

2.1 First Step to Plan Human Disease Modeling Experiments

This is one of the most critical steps when setting out to plan a human disease modeling experiment. Different starting materials will be necessary, depending on whether the question can be best answered by performing genome editing in human pluripotent stem cells or patient-derived material. In the latter, somatic cells (fibroblasts, blood, others) will be needed for reprogramming into induced pluripotent stem cells or transdifferentiation/generation of induced neurons (for further details see Sect. 2.2.1). A check for respective rules of the Institutional Review Board and obtaining the necessary permissions according to the governmental regulations are a must.

2.2 Human Stem Cell-Based Disease Models

2.2.1 Transdifferentiation

Transdifferentiation, or lineage reprogramming, is the conversion of somatic cells into a cell of a different somatic lineage without passing the intermediate stage of pluripotency (Fig. 1). The force to change identity is achieved by ectopic expression

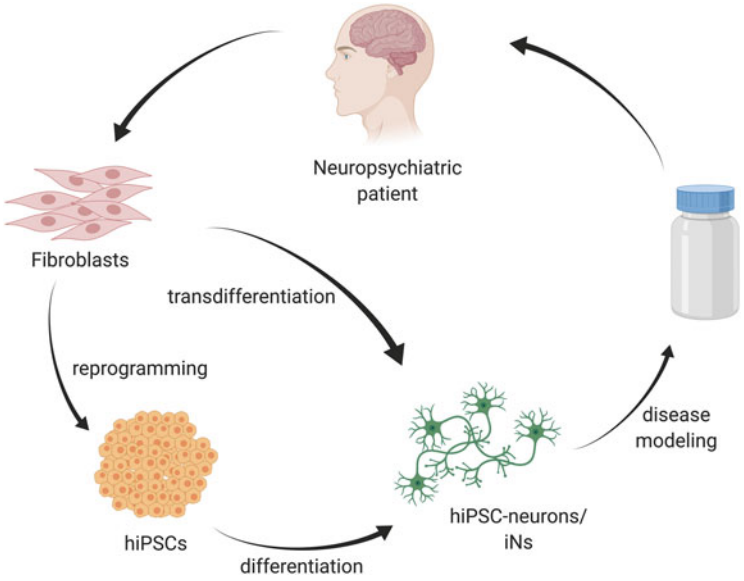

Fig. 1 Strategy for the generation of stem cell-based neural disease models. Patient-derived somatic cells are transdifferentiated into iNs or reprogrammed into hiPSCs and subsequently differentiated into disease-relevant cell types. Based on in vitro disease modeling, treatment of patients can be personalized in the future. Created with Biorender.com

of tissue-specific transcription factors (Davis et al. 1987). This switch to another cell type is considered to have great promise for regenerative medicine. For neuropsychiatric disease, especially disease modeling and compound testing are potential applications. Human fibroblasts, due to accessability and easy in vitro expansion, are frequently the cell type of choice for direct conversion into functional induced neurons (iNs), though hepatocytes or adipocytes have been successfully reprogrammed (Colasante et al. 2015; Vierbuchen et al. 2010). For example, a combination of the transcription factors achaete-scute homolog 1 (ASCL1), neurogenin 2 (NGN2), neurodifferentiation D1 (NEUROD1), POU domain class 3 transcription factor 2 (POU3F2), and myelin transcription factor 1-like protein (MYT1L) are required for efficient conversion into iNs (Mertens et al. 2016; Pang et al. 2011; Pfisterer et al. 2011; Son et al. 2011; Vadodaria et al. 2016). Moreover, computational approaches are made to predict transcription factors for developing transdifferentiation protocols (Rackham et al. 2016). The time-saving technique of lineage reprogramming into mature cells compared to pluripotent reprogramming could be applied faster for compound screening (Mertens et al. 2016). The applicability of direct lineage reprogramming of patient-derived cells for disease modeling has been shown for Alzheimer's disease (AD) (Hu et al. 2015). The major advantages of transdifferentiation are the fast speed of generating a human neuron and that the epigenetic landscape and aging signatures are preserved (Mertens et al. 2015a, b). However, limiting factors of iNs are the huge amount of needed patient material, the genetic mosaicism, and maturity of iN cultures (Gascón et al. 2017; Mertens et al. 2016).

2.2.2 Human Embryonic Stem Cells

In 1998, Thomson et al. described the first isolation of human embryonic stem (ES) cells derived from the blastocyst stage of a human preimplantation embryo. The self-renewable human ES cells were characterized as pluripotent and have the potential to differentiate into all three embryonic germ layers (Thomson et al. 1998). Human ES cells have been differentiated toward various neuronal cell fate and opened new possibilities to study early human neurodevelopment and pathological mechanisms causing neuronal disorders (Chambers et al. 2009; Kriks et al. 2011; Maroof et al. 2013; Nicholas et al. 2013; Perrier et al. 2004). ES cells carrying specific mutations can be obtained from embryos identified by preimplantation genetic diagnosis (PGD) (Eiges et al. 2007; Niclis et al. 2013). The classical generation of ESC-based disease models was done via viral expression or knockdown of specific genes (Urbach et al. 2004, 2009).

Even though human ES cells represent a defined cell type where huge experience exists also for differentiation protocols, governmental restrictions and ethical controversies need to be considered when planning an experiment with human ES cells. However, with the establishment of new genome editing methods, disease modeling based on human ES cells became more popular in the past years (Avior et al. 2017; Dever et al. 2016; Fogarty et al. 2017; Li et al. 2013; Liao et al. 2015; Soldner et al. 2016).

2.2.3 Human-Induced Pluripotent Stem Cells

The difficulty of limited availability and ethical controversy using human embryos was resolved by the discovery to jump-start pluripotency by overexpression of the transcription factors OCT3/4, SOX2, KLF4, and MYC (Takahashi et al. 2007). Human somatic cells can be reprogrammed to induced pluripotent stem (iPS) cells by transient expression of the pluripotency-specific transcription factors. For the first time, fibroblasts directly isolated from patients were reprogrammed and characterized for induced pluripotency and differentiation potential (Park et al. 2008). The patient-specific iPS cell lines offer a great opportunity for disease modeling of human brain disorders in vitro (Fig. 1), supported by the abundant availability of reliable differentiation protocols for various neuronal subtypes. Major discoveries for neuropsychiatric diseases were made through modeling of complex psychiatric disorders SCZ, ASD, and BPD using reprogrammed patient iPS cells (Brennand et al. 2011; Mertens et al. 2015b; Marchetto et al. 2010; Robicsek et al. 2013). Prior to planning an iPS cell-based experiment, one needs to think of the minimal number of patients and cell lines per patient, for example, including at least three diseased and three probands. The major advantage of iPS cells is the opportunity to generate patient-specific stem cells and develop personalized medicine for both genetic and sporadic diseases (Fig. 1). On the other hand, high variability, low reproducibility, and the choice of respective controls challenge stem cell-based experiments. Isogenic controls of patient-specific iPS cells could be a profitable approach enabled by genome editing.

2.2.4 Genome Editing

With the focus on monogenic disorders, genome editing can either be used to induce disease-causing mutations in healthy control cells or correct patient-specific genetic variants and generate isogenic control cell lines. Transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) have been utilized to target specific DNA sequences in human pluripotent stem cells (Hockemeyer et al. 2017; Soldner et al. 2011). With the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated 9 (Cas9) system, a revolutionary opportunity for disease modeling has emerged (Jinek et al. 2012). The CRISPR/Cas9 system efficiently targets and cleaves desired DNA sequences, previously also shown for human iPS cells (Mali et al. 2013). Because of the remaining risk of potential indel formations at off-target sites in the genome, due to imperfect complementary of the guide RNA, recent studies used Cas9 mutants to increase the specificity of genome editing (Slaymaker et al. 2016; Chen et al. 2017). Unwanted and undetected off-targets in iPS cells endanger the reliability of new genome editing methods and still need to be optimized for preclinical studies. However, the major advantage of the CRISPR/Cas9 system is the possibility to perform rescue experiments and generate isogenic control cell lines that reduce the heterogeneity of iPS cell cultures and increase the reproducibility of stem cell-based experiments.

2.3 Analysis of Disease-Relevant Phenotypes

2.3.1 The "-OMIC" Tools for Disease Modeling

GWAS, whole exome sequencing (WES) studies, and copy number variation (CNV) studies of large patient cohorts have identified a range of genetic risk factors and biological pathways associated with mental disorders, such as ASD, BPD, SCZ, major depressive disorder, and attention-deficit hyperactivity disorder (ADHD) (Lee et al. 2013; Purcell et al. 2014; Ripke et al. 2013, 2014). Only a small percentage of psychiatric disorders are caused by single-gene mutations, for instance, Rett syndrome, Timothy syndrome, and familial AD (fAD). Hence, patient-derived iPS cells represent an efficient way to study the disease-relevant phenotypes of sporadic psychiatric disorders on the cellular and molecular level. Next-generation sequencing technologies identified altered gene expression in patient-derived iPS, neural progenitor cells (NPCs), and neurons (Brennand et al. 2011; Lin et al. 2014; Madison et al. 2015; Paşca et al. 2011; Wang et al. 2015; Wen et al. 2014). RNA-Seq analysis can also be achieved on single-cell level and could be used to identify molecularly distinct cell types (Bardy et al. 2016; Camp et al. 2015; Quadrato et al. 2017).

However, disease-causing expression profiles might as well result from epigenetic changes. Since the reprogramming process is thought to remove many epigenetic modifications, diseases with solely epigenetic causes might be difficult to study in human iPS cells. Previous studies have shown that iPS cells retain an epigenetic memory from their somatic origin, suggesting hot spots in the genome with unknown function that escape from epigenetic reprogramming (Kim et al. 2010; Lister et al. 2011; Nishino et al. 2011). The influences of epigenetic profiles of DNA methylation or histone modifications on the pathology of psychiatric disorders can be analyzed among others by chromatin immunoprecipitation sequencing (ChIP-Seq) (Brennand et al. 2015; Sheridan et al. 2011; Sugathan et al. 2014; Urbach et al. 2010). Nonetheless, the discussion about the epigenetic landscape after reprogramming and transdifferentiation is still ongoing. During reprogramming, closed and compacted chromatin of a somatic cells acts as a barrier for the complete establishment of pluripotency and an epigenetic landscape that is similar to ES cells. On the other hand, transdifferentiation combines downregulation of donor cell-specific genes and upregulation of target cell-specific genes and preserves certain epigenetic modifications (Qin et al. 2016). For disease modeling, one needs to keep in mind that direct conversion of somatic cells skips developmental precursor cell stages, and therefore one might miss associated phenotypes. Therefore, neurodevelopmental diseases such as ASD and Rett syndrome should be studied using iPSC-derived NPCs and neurons (Fig. 2). On the other hand, neurodegenerative diseases in certain circumstances might be better off with transdifferentiated iNs, because of the preserved age-related epigenetic signatures (Mertens et al. 2015a, b).

Lastly, quantitative proteomic mass spectrometry using stable isotope labeling by amino acids in cell culture (SILAC) of disease-relevant cell types and subsequent

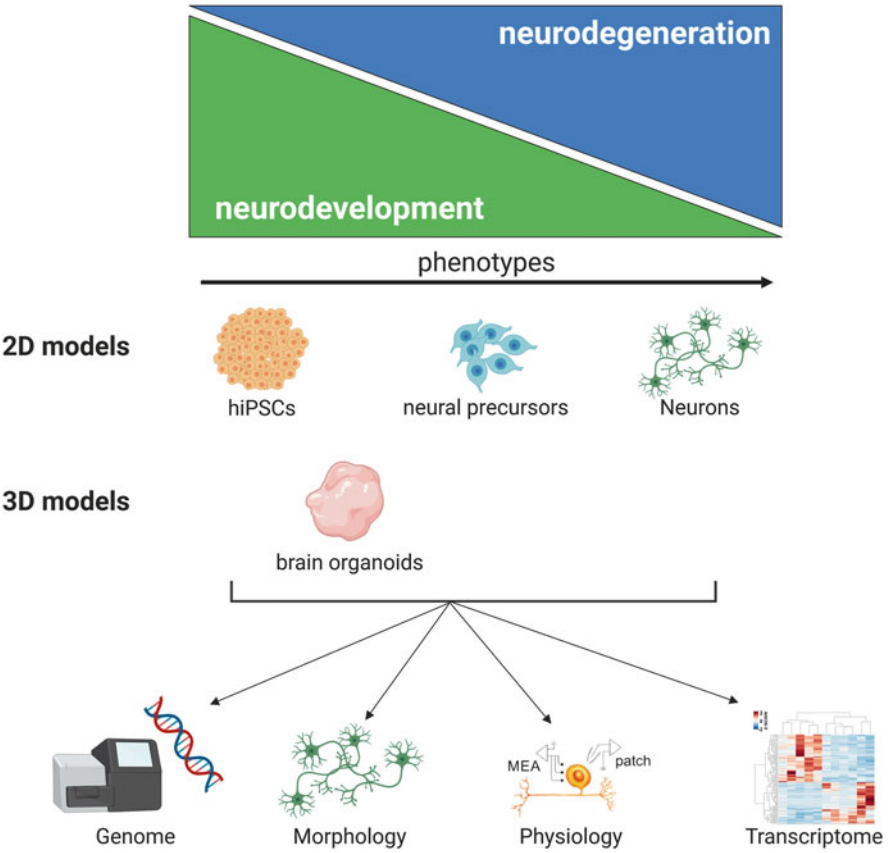

Fig. 2 Current approaches of stem cell-based neural disease models for neuropsychiatric disorders. Modeling phenotypes of neurodevelopmental and neurodegenerative diseases is partially restricted to developmental cell stages. Current analysis of patient-derived iPS cells and neural cells offers a range of potential methods, comprising genome, morphology, physiology, and transcriptome. Created with Biorender.com

network analyses detected abnormal protein levels and affected pathways in patients (Brennand et al. 2015; Li et al. 2015; Tobe et al. 2017). These robust and quantifiable techniques enable reproducible high-throughput approaches and can reveal biological pathways associated with psychiatric disorders in a human-based cellular model (Fig. 2).

2.3.2 Cellular Deficits in Neurodevelopment

Changes on the (epi-) genetic, transcriptional, and protein level are indicative of disturbed pathways in patients suffering from psychiatric disorders. The ideal

workflow of stem cell-based models is the generation of authentic and reproducible cell types to mimic the aspects of the human central nervous system (CNS). Differentiating human iPS cells into NPCs and neuronal subtypes can recapitulate the brain development in vitro and further unveil disease-relevant cellular phenotypes. In addition to the choice of respective patients, it is critical to identify the appropriate cell type to study. The aim of numerous differentiation protocols is the efficient generation of homogenous cell systems, for example, toward glutamatergic, GABAergic, dopaminergic, and motor neurons, to enable the study of neurotransmitter-specific phenotypes (Wen et al. 2016). Moreover, it needs to be considered at which developmental cell stage the phenotype might be observable. Phenotypes of age-related diseases, such as AD, are more likely detectable in mature neurons, whereas neurodevelopmental phenotypes of psychiatric diseases might already be observed at precursor cell stages (Fig. 2). For neuropsychiatric disorders, several neurodevelopmental phenotypes have been observed during the differentiation process of patient-derived iPS cells into NPCs and neurons. The detected morphological phenotypes of human stem cell-based models for psychiatric diseases include smaller neuronal somas, reduced number of spines, and decreased neurite number and neurite lengths (Brennand et al. 2011; Cheung et al. 2011; Doers et al. 2014; Marchetto et al. 2010; Sheridan et al. 2011).

Moreover, some disease-specific phenotypes are already visible at the iPSC or NPC stage of in vitro cultivation. Altered proliferation, length of cell cycle, or deficits in the cytoskeleton organization of patient-derived iPS cells and NPCs were found in disease models of several psychiatric disorders, furthermore supported by gene expression analyses (Madison et al. 2015; Marchetto et al. 2017; Mariani et al. 2015; Yoon et al. 2014). Developmental deficits of iPSC-derived neurons in terms of differentiation efficiency and neuronal maturation found in vitro can give insights into the in vivo neurodevelopment of patients affected by psychiatric disorders (Madison et al. 2015; Robicsek et al. 2013). Considering the heterogeneous and complex genetic background of mental disorders, it is important that cellular phenotypes are robust and reproducible. For instance, a stem cell-based experiment should include three or more probands and controls with various lines from each individual. Human pluripotent stem cell-derived neural cells are in conclusion a relevant tool to model the complex pathology of psychiatric disorders.

2.3.3 Functionality of Neuronal Networks

Connecting genetic and transcriptomic results with developmental phenotypes observed during in vitro differentiation of patient-derived cells can give an important hint toward pathways involved in psychiatric diseases. An additional contribution to the patients' phenotypes concerns the functional metabolism and excitability of neurons and their interactions with glia cells. Metabolic abnormalities like oxidative stress or mitochondrial deficits were, for example, found in patients suffering from SCZ (Brennand et al. 2015; Paulsen BDa et al. 2012; Robicsek et al. 2013). Since synapses are the major communication points, disruption of synaptic connections

might induce mental disorders in humans. Impaired synaptic activity in patients was identified using stem cell-based models, including defects in neurotransmitter release (Hook et al. 2014; Wen et al. 2014; Yu et al. 2014). Hyperexcitability and hyperactivity of patient-derived neurons were furthermore detected for BPD using patch-clamp recording and Ca^{2+}-imaging (Mertens et al. 2015b).

Furthermore, synaptic connections within the human brain are vulnerable to disruption and might trigger neuropsychiatric disorders. Decreased neuronal connectivity provoked by reduced synaptic connections and synaptic density in SCZ has been found in several postmortem studies and was further shown in iPS cell-derived neurons (Brennand et al. 2011). Even though NPCs and neurons derived from SCZ patients showed no apparent defects of protein expression and synaptic functionality or density, neuronal connectivity was disrupted. Interestingly, Brennand et al. took advantage of an assay that chases transneuronal spread of rabies which is usually transferred via synaptic contacts and hereby demonstrated reduced connectivity in patient-derived neurons compared to control neurons (Brennand et al. 2011). The multielectrode arrays (MEA) system is becoming an increasingly relevant tool to analyze neuronal activity and connective networks of iPS-derived neurons in vitro, demonstrating their capacity to self-organize into spontaneous and synchronous active networks (Xu et al. 2017). Moreover, MEA systems might be a useful tool to identify neurons that exhibit pacemaker properties and trigger synchronous activity of the neuronal network in a human cellular system (Illes et al. 2014).

Interactions in the human brain do not only occur between neurons. Glia cells represent the predominant brain population; therefore neuroglia interactions might have an important contribution to psychiatric diseases. Glia-associated phenotypes in psychiatric patients were found for different glial cells including astrocytes, oligodendrocytes, and microglia, indicating a higher complexity of the disease-causing mechanisms in the human brain (Cotter et al. 2001; Yamamuro et al. 2015). Abnormalities of all three glia cells are implicated, for example, in the disease mechanism of SCZ (Bernstein et al. 2015). (Non-) autologous co-culturing of patient-derived neurons and glia cells represent a valid system to investigate the influence and interaction between the neural subtypes. Differentiation of patient-derived iPS cells into *astrocytes* further indicated a crucial disease mechanism of glia cells in psychiatric disorders (Russo et al. 2017; Williams et al. 2014). Even though several protocols have been established to generate iPS cell-derived astrocytes, their implementation in disease modeling is still ongoing (Krencik et al. 2011; Shaltouki et al. 2013; Tcw et al. 2017). Early cell-cell interactions are required for astrocyte maturation, limiting the in vitro differentiations. Moreover, culture conditions in co-culture systems should be specially tailored to the physiological characteristics of astrocytes (Tcw et al. 2017). Since *microglia* are essential for physiological brain functionality, associations with psychiatric phenotypes were found (Derecki et al. 2012). Several approaches have been made to generate microglia out of human iPS cells (Abud et al. 2017; Douvaras et al. 2017; Muffat et al. 2016; Pandya et al. 2017) or to transdifferentiate human monocytes (Etemad et al. 2012; Ohgidani et al. 2017). Differentiation of human iPS cells into microglia-like cells is a major challenge due to their unique developmental origin. However, glial dysfunctions represent a

hallmark of neuropsychiatric disorders, and including glia into new cell models might give new insights into their contribution to patients' phenotypes.

2.3.4 Neuronal Network Interactions in Three-Dimensional (3D) Cell Systems

Co-culture systems of brain cells are usually performed in an adherent and two-dimensional (2D) culture, lacking the 3D structure and cell-cell interactions those found in vivo. Moreover, the characteristics of neuropsychiatric disorders usually relate to reduced connectivity and circuit dysfunction. The shift from single-cell measurements to multidimensional analyses by use of brain organoids generated from human iPS cells revolutionized disease modeling of brain disorders (Lancaster et al. 2013). Brain organoids grown in suspension recapitulate specific features of the developing brain, including incorporation of various neural subtypes and spatial organization. Adding brain region-specific patterning factors to the organoid differentiation can form a more homogenous cell population than unpatterned organoids. Recently, 3D models of developing hippocampus (Sakaguchi et al. 2015), midbrain (Jo et al. 2016; Qian et al. 2016), forebrain (Birey et al. 2017), hypothalamus (Qian et al. 2016), and cerebellum (Muguruma et al. 2015) have been generated. On the contrary, unpatterned whole-brain organoids generate tissues with a more heterogeneous cell composition, as shown by immunohistochemistry and single-cell RNA sequencing (Camp et al. 2015; Lancaster et al. 2013; Quadrato et al. 2017). Moreover, neuronal cell migration within 3D cell systems has been studied through the fusion of region-specific brain organoids, suggesting high potential for robust neuronal network studies in human stem cell-based organoids (Bagley et al. 2017). Since the field is still young, available data for modeling psychiatric disorders using brain organoids is limited (Mariani et al. 2015; Wang et al. 2017). So far, 3D brain organoids have the greatest potential to model cell-cell-interactions with broad cellular diversity. Moreover, incorporation of glia cells into the organoids might promote the neuronal and circuit maturation that are impaired in neuropsychiatric disorders.

2.4 Examples for Stem Cell-Based Disease Modeling of Neuropsychiatric Disorders

Subsequently, exemplary studies on ASD (Table 1) and AD (Table 2) will be chosen, and major results of disease modeling experiments performed with the previously mentioned methods will be described.

Table 1 Current stem cell-based disease models of ASD and their major findings

Autism spectrum disorder	Phenotype	Major result	hPSCs	NPCs	Neurons	3D models
	Genome/ epigenome	Maintenance of modifications upon differentiation	Sheridan et al. (2011), Sugathan et al. (2014), Urbach et al. (2010), Mariani et al. (2015), Marchetto et al. (2010), Cheung et al. (2011) and Doers et al. (2011)		Marchetto et al. (2010)	
	Transcriptome	Differential expression of neurodevelopmental genes	Marchetto et al. (2017)	Sugathan et al. (2014), Paşca et al. (2011), Wang et al. (2015), Marchetto et al. (2017) and Wang et al. (2017)	Paşca et al. (2011), Wang et al. (2015), Marchetto et al. (2017), Wang et al. (2017), Shcheglovitov et al. (2013) and Baldino-Russo et al. (2017)	Mariani et al. (2015) and Wang et al. (2017)
	Proliferation/ differentiation	Altered proliferation at NPC stage	Mariani et al. (2015)	Marchetto et al. (2017) and Mariani et al. (2015)	Pasca et al. (2011), Mariani et al. (2015), Shcheglovitov et al. (2013) and Baldino-Russo et al. (2017)	Mariani et al. (2015)
	Neurite growth/ synaptogenesis	Reduced synaptogenesis and neurite outgrowth			Sheridan et al. (2011), Marchetto et al. (2017), Mariani et al. (2015), Marchetto et al. (2010), Cheung et al. (2011), Doers et al. (2011), Shcheglovitov et al. (2013) and Baldino-Russo et al. (2017)	Mariani et al. (2015)
	Electrophysiology	Reduced connectivity and network activity			Marchetto et al. (2010), (2017), Mariani et al. (2015),	Mariani et al. (2015)

(continued)

Table 1 (continued)

					Shcheglovitov et al. (2013) and Baldino-Russo et al. (2017)	
	Biochemical	Rescue of cellular phenotype		Paşca et al. (2011) and Marchetto et al. (2017)	Pasca et al. (2011), Marchetto et al. (2010), (2017), Shcheglovitov et al. (2013) and Baldino-Russo et al. (2017)	Mariani et al. (2015)
	Glia cells	Astrocytes contribute to neuronal phenotype	Sheridan et al. (2011), Baldino-Russo et al. (2017) and Williams et al. (2014)			

Table 2 Current stem cell-based disease models of AD and their major findings

Alzheimer's disease	Phenotype	Major result	hPSCs	NPCs	Neurons	3D models
	Genome/ epigenome					
	Transcriptome	Differential expression correlates with dysregulation in AD brains	Sproul et al. (2014)	Sproul et al. (2014)	Kondo et al. (2013)	
	Proliferation/ differentiation					
	Neurite growth/ synaptogenesis					
	Electrophysiology	Altered excitatory synapse activity			Honda et al. (2016)	
	Biochemical	Increased Aβ42/40 ratio and tau phosphorylation		Liu et al. (2012), Sproul et al. (2014)	Kondo et al. (2013), Israel et al. (2012), Yagi et al. (2011), Muratore et al. (2014), Choi et al. (2014), Raja et al. (2016), Shi et al. (2014), Liu et al. (2012), Koch et al. (2012), Sproul et al. (2014) and Ochalek et al. (2017)	Choi et al. (2014) and Raja et al. (2016)
	Glia cells	Aβ peptides in astrocytes, increased oxidative stress in astrocytes	Kondo et al. (2013) and Oksanen et al. (2017)			

2.4.1 Autism Spectrum Disorder

ASD is a behaviorally defined condition that is characterized by a broad spectrum of mild to severe disabilities in communication, social interaction, and stereotyped repetitive behavior. Only a minority of ASD cases is monogenic. However, GWAS of large patient cohorts identified numerous rare genetic variants associated with ASD (Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium 2017). The primary pathological cause of ASD is thought to be altered neurodevelopment, leading to reduced neuronal circuitry, abnormal synaptogenesis, and dysregulation of the excitatory/inhibitory (E/I) balance (Persico et al. 2006). Nonetheless, ASD patients are phenotypically and etiologically heterogeneous, making it challenging to unveil the cellular mechanisms. Human iPS cell-derived neurons have been generated from monogenic ASD patients carrying mutations in MeCP2 (Rett syndrome), CACNA1C (Timothy syndrome), and SHANK3 (Phelan-McDermid syndrome), showing synaptic defects, altered calcium signaling, and electrophysiological aberrations (Marchetto et al. 2010; Paşca et al. 2011; Shcheglovitov et al. 2013). However, uncovering the phenotypes of complex ASD forms remains challenging.

A recent study of Marchetto et al. (2017) showed that early enlarged brain volume of sporadic ASD patients correlated with increased proliferation of iPS cell-derived NPCs. Based on genomic analyses, the altered proliferation of ASD NPCs resulted from changes in the canonical Wnt signaling pathway. Upon neuronal differentiation, they found a significant reduction of inhibitory neurons and impaired synaptogenesis. Neuronal network analysis of ASD neurons showed connectivity defects that might result from the observed imbalance of excitatory/inhibitory neurons and was further supported by RNA sequencing. In this study, patient-specific NPCs and neurons were utilized to examine the underlying molecular mechanisms of early brain enlargement in ASD patients (Marchetto et al. 2017).

Furthermore, early cortical development in patients with idiopathic ASD was modeled combining RNA sequencing and generation of iPS cell-derived telencephalic organoids. The transcriptome analysis of ASD patients revealed altered transcriptional regulation of cell proliferation, neuronal differentiation, and synaptic transmission. Subsequent analysis confirmed a significant decrease in cell cycle length of ASD-derived iPS cells and increased neuronal and synaptic differentiation in patient-specific organoids. Neuronal cell fate was altered in ASD-derived organoids, resulting in an imbalance between glutamatergic and GABAergic neurons. These differences were caused by an overexpression of the transcription factor FOXG1; knockdown of FOXG1 rescued the imbalance of GABA/glutamate neurons. Additionally, FOXG1 expression correlated with autism symptom severity of the patients, proposing a potential biomarker for sporadic ASD. This study emphasizes the importance of patient-specific disease modeling of idiopathic ASD and identifying molecular and cellular alterations using stem cell-based organoids (Mariani et al. 2015).

2.4.2 Alzheimer's Disease

In contrary, a psychiatric disorder that appears at advanced age is AD, affecting approximately 30 million people worldwide. AD is clinically characterized by progressive loss of memory, learning disability, and increasing difficulties in daily tasks with varying severity of neuropsychiatric symptoms. The pathological hallmarks of AD are extracellular plaques composed of insoluble amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles formed by hyperphosphorylated tau protein associated with loss of neurons and synapses in the cerebral cortex and subcortical regions. Early-onset fAD is primarily caused by mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes and accounts for up to 5% of all AD cases. Modeling AD using human iPS cells was initiated with familial cases, caused by specific point mutations in the *APP*, *PSEN1*, or *PSEN2* gene. The patient-derived NPCs and neurons showed elevated secretion of longer Aβ peptides and altered expression and phosphorylation of tau compared to healthy control NPCs and neurons (Israel et al. 2012; Koch et al. 2012; Kondo et al. 2013; Liu et al. 2014; Muratore et al. 2014; Yagi et al. 2011; Sproul et al. 2014). Recently, elevated Aβ production and tau hyperphosphorylation were also shown for iPS cell-derived neurons derived from sporadic AD patients (Ochalek et al. 2017). Due to variability in differentiation efficiency, Israel et al. performed a fluorescence-activated cell sorting (FACS) step prior to differentiation and purification of NPCs. The accumulation of Aβ peptides was also found in adults suffering from Down syndrome caused by trisomy 21, due to increased expression of APP which is encoded on chromosome 21 (Shi et al. 2012). Overexpression of mutant *PSEN1* resulted in synaptic dysfunction of human ES cell-derived neurons, indicating additional disease-causing mechanisms besides protein aggregates (Honda et al. 2016). Kondo et al. were able to support the hypothesis that endoplasmic reticulum (ER) and oxidative stress, induced by Aβ aggregates, are associated with the pathogenesis of AD, in both familial and sporadic cases. Increased oxidative stress in astrocytes might also play an important role in the pathogenesis of AD, as indicated by previous studies (Kondo et al. 2013; Oksanen et al. 2017). However, a common challenge of modeling AD in vitro is the optimal timeframe to observe relevant phenotypes of age-related diseases (Fig. 2). Brain organoids generated from fAD patients that recapitulated AD pathology showed progressive increase in number and size of Aβ aggregates over time compared to control organoids (Choi et al. 2014; Raja et al. 2016). Therefore, organoids seem to be an important stem cell model to investigate age-related AD pathology in a time-dependent manner. Moreover, interference in the APP processing by inhibition of β- and γ-secretase decreased the number of Aβ aggregates, indicating that organoids might be a relevant model for future compound testing.

2.5 *Untangling the Relationship Between Genotype and Phenotype*

The increasing abundance of phenotypic data for neurological disorders of studies using human iPS cells makes it more and more difficult to correlate underlying mechanisms. A recent meta-analysis systematically searched 93 published articles, reporting neurological phenotypes of 31 neurological diseases using patient-specific cells, to unravel relationships between phenotypes and genotypes (Hollingsworth et al. 2017). With the iPS cell phenogenetic map project atlas (iPhemap), the accrued information is available online and will be continually updated with patient-derived models of neurodevelopmental and neurodegenerative diseases. Most of the included studies investigated neurodegenerative diseases with characterized somatic mutations (67%), followed by neurodevelopmental diseases (30%). Interestingly, differences were found in research practices and reporting of methods. Only a few studies used isogenic controls, and only 35% of the studies were conducted with three or more patient cell lines. Grouping of the observed phenotypes by a defined algorithm revealed increased or decreased cellular processes or products in 70% of the phenotypes included into the meta-analysis. Moreover, patient-derived oligodendrocytes and neurons were cell types with the largest number of reported phenotypes. The phenotype class "rescue/recovery from disease phenotypes after chemical treatment" was also mainly observed in neurons, indicating cell type-specific phenotypes in disease modeling. These findings were supported by the fact that neurodegenerative disease phenotypes were predominantly found at the neuronal stage, whereas neurodevelopmental phenotypes can be identified at early stages of differentiation (Fig. 2).

Network analysis of the phenotypes revealed novel gene ontology (GO) associations for several diseases and overlapping phenotypes between different diseases. Furthermore, the researchers included transcriptome data to determine the relationship between molecular and cellular phenotypes. Correlation between the molecular abnormalities and cellular phenotypes in iPS cells was found for neurodevelopmental disorders, implying an association between the molecular and cellular phenotypes. However, no correlation was found for neurodegenerative diseases. Significant molecular aberrations were identified at the neuronal level of both neurodevelopmental and neurodegenerative disorders, reflecting the cellular phenotypes observed by the included studies and implying high phenogenetic correlation. Taken together, iPhemap illustrates novel opportunities how phenogenetics can be used to improve future modeling of neurological disorders.

3 Challenges and Outlook

A large proportion of the worldwide population is affected by several neuropsychiatric disorders. Even though human iPS cells provide a broad platform for disease modeling of monogenic and idiopathic disorders, there are several technical

challenges that limit the applicability of stem cell models. A major concern is the high variability between different patients or even between different clones of the same patient. Standardized protocols for reprogramming, cell culture, and differentiation are required for reproducible cell-based assays. A panel of control cell lines are necessary to determine disease-specific phenotypes. However, since human iPS cell culture and differentiation into disease-specific cell types is time-consuming and highly expensive, the sample size is often kept to a minimum.

Secondly, early cell-cell interactions are required for complete maturation of neurons and glia cells, missing in cell type-specific cultures and limiting disease modeling of whole-brain disorders like psychiatric conditions. Co-culture systems and brain organoids are expected to be a more suitable model to uncover connectivity phenotypes, especially in psychiatric disorders which are induced by altered interaction and communication between various cell types from different brain regions. Nonetheless, iPSC-derived brain organoids show high heterogeneity, variable structures, and cell composition, warranting optimization of current protocols.

Another concern in disease modeling using iPS cells that resemble fetal cell stage are questions relating to late-onset or neurodegenerative diseases, and that might be caused by aging and environmental factors. Variable possibilities have been proposed to overcome this challenge and accelerate the appearance of pathological phenotypes in cell culture, for instance, by exposure of the cells to environmental factors like oxidative stress or manipulation of intrinsic mechanisms (Miller et al. 2017; Vera et al. 2016). Finding new strategies to model the effects of aging and long-term environmental influences could reveal new pathological phenotypes of neurodegenerative disorders in the future.

Stem cell-based models represent a big opportunity for applications in translational medicine, providing a novel platform for discovering personalized therapies for patients and potential biomarkers to monitor the patients' disease progression. Developing technologies of automated cell culture on a large scale enables high-throughput compound testing. However, several aspects need to be considered prior to cell-based drug screening. For therapeutic implications, the patient-derived iPS cells should be well characterized genetically and phenotypically. Furthermore, choosing correct and homogeneous cell type and phenotype for measuring drug response is highly relevant. Nonetheless, previous studies successfully identified potential drugs for psychiatric disorders that were able to rescue the patients' phenotype (Brennand et al. 2011; Marchetto et al. 2010; Paşca et al. 2011; Shcheglovitov et al. 2013). Moreover, transplantation of iPS cells and derivates into diseased human tissue comes more and more into focus, though cell replacement therapy of neurological disorders is extremely challenging.

In conclusion, future stem cell-based disease modeling will generate a wealth of information about human neuropsychiatric disorders. With the recently developed techniques of cell reprogramming, differentiation protocols, genome editing, and high-throughput sequencing, previous studies represent only a fraction of the possibilities of patient-derived iPS cells in disease modeling and drug discovery. While there are still technical challenges that require improvement, the ability to investigate neuropsychiatric diseases in vitro at single-cell resolution and in 3D cell systems

demonstrates that human stem cells are a powerful tool to promote preclinical studies in the scientific future.

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Role of MicroRNAs in Anxiety and Anxiety-Related Disorders

Conor P. Murphy and Nicolas Singewald

Contents

Abstract MicroRNAs as critical regulators of gene expression important for functions including neuronal development, synapse formation, and synaptic plasticity have been linked with the regulation of neurobiological systems that underlie anxiety processing in the brain. In this chapter, we give an update on associative evidence linking regulation of microRNAs with anxiety- and trauma-related

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disorders. Moving beyond correlative research, functional studies have emerged recently that explore causal relationships between microRNA expression and anxiety-like behavior. It has been demonstrated that experimental up- or downregulation of the candidate microRNAs in important nodes of the anxiety neurocircuitry can indeed modulate anxiety-related behavior in animal models. Improved methodologies for assessing microRNA-mediated modulation have aided such functional studies, revealing a number of anxiety-regulating microRNAs including miR-15a, miR-17-92, miR-34, miR-101, miR-124, miR-135, and miR-155. Important functional target genes of these identified microRNAs are associated with specific neurotransmitter/neuromodulator signaling, neurotrophin (e.g., BDNF) expression and other aspects of synaptic plasticity, as well as with stress-regulatory/hypothalamic-pituitary-axis function. Furthermore, microRNAs have been revealed that are regulated in distinct brain regions following various anxiety-attenuating strategies. These include pharmacological treatments such as antidepressants and other drugs, as well as non-pharmacological interventions such as fear extinction/exposure therapy or positive stimuli such as exposure to environmental enrichment. These are first indications for a role for microRNAs in the mechanism of action of anxiolytic treatments. As research continues, there is much hope that a deeper understanding of the microRNA-mediated mechanisms underlying anxiety-related disorders could open up possibilities for future novel biomarker and treatment strategies.

Keywords Anxiety disorders · Anxiolytic · Epigenetics · microRNA · RDoc

1 Introduction

Physiological anxiety is a beneficial emotion in response to real or potential threats, but patients with pathological anxiety display a broad range of exaggerated and/or enduring symptoms, such as increased arousal/vigilance and excessive fear- and anxiety-related responses (for detailed discussion into differences and characteristics between fear and anxiety, see Perusini and Fanselow 2015) including autonomic changes such as heart rate increases and sweating, often in the absence of any real threat or danger (Dell'osso et al. 2010). Many of these symptoms are shared by different anxiety disorders, which are classified in the tenth edition of the International Classification of Diseases (ICD-10) (WHO 1993; Craske et al. 2017) and the fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) (American Psychiatric Association 2013). They include specific phobias, panic disorder (with or without agoraphobia), social phobia, and generalized anxiety. Anxiety- and trauma-related disorders, such as post-traumatic stress disorder (PTSD), are grouped together in the ICD-10 (WHO 1993; Craske et al. 2017). The current diagnostic systems are based on subjective measures of client self-report, clinical observation, and clinical judgment, while the recently proposed Research Domain Criteria (RDoC) system defines psychopathologies as phenomena of multilevel neurobiological characteristics underlying dimensions of behavior from normal to abnormal (Anderzhanova et al. 2017). The RDoC system holds promise

for better characterization of complex psychiatric disorders that are commonly comorbid, as in the case of anxiety often comorbid with depression and alcohol/drug dependence, and aims to improve translatability of studies from animals to humans by supporting the endophenotype-based comparison on a neurobiological basis across behavioral dimension (Anderzhanova et al. 2017). Anxiety-related disorders are the most common mental disorders, affecting up to 14% of Europeans each year (Wittchen et al. 2011). In developed countries, the lifetime prevalence of these disorders is up to 28% (Kessler et al. 2012). Anxiety disorders impact the quality of life and place a high emotional and financial burden on families and government bodies (Olesen et al. 2012; Wittchen et al. 2011). Furthermore, the high comorbidity that exists between anxiety and other psychiatric disorders, such as depression and bipolar disorder, makes treating these disorders even more complex (Pavlova et al. 2017; McMillan et al. 2017).

Both psychotherapy and pharmacotherapy are currently used to treat anxiety-related disorders and have been shown to be more effective than placebo. However, a considerable percentage of patients only exhibit partial long-term benefits following therapy, failing to achieve complete remission (Bandelow et al. 2007, 2012; Farach et al. 2012). Pharmacological treatments include selective serotonin reuptake inhibitors (SSRIs) and other antidepressants (Baldwin et al. 2014). Benzodiazepines are not recommended as first-line treatments due to their potential side effects such as increased risk of drug dependence and should be used only transiently. Disadvantages of the current first-line drug treatments include a delayed period before the onset of therapeutic benefits, suicidal tendencies, and risk of sexual dysfunction, often leading to discontinuation of treatment (Maron and Nutt 2015; Farb and Ratner 2014; Ravindran and Stein 2010). Hence, we are in need of novel interventions targeting causal factors and resulting in long-lasting benefits with lower incidence of side effects. Despite this clear need for improved treatments, the development of novel drugs for anxiety-related disorders has been rather unsuccessful (Singewald et al. 2015; Griebel and Holmes 2013). One of the most important factors involved in this is the still limited knowledge that exists about underlying mechanisms and pathways mediating and modulating anxiety-related behavior, although impressive advances have been made.

2 Neurobiology and Molecular Basis of Anxiety

Key to understanding anxiety processing in the brain is the elucidation of the neuronal correlates that are involved. Regulation of fear and anxiety behavior depends on neuronal activity and communication between relevant brain areas, and there is evidence that cellular plasticity is vital for appropriate inter-regional communications (Hill and Martinowich 2016). It has been found that there is a high degree of overlap between humans and animals in the neural circuitry underlying anxiety-related behaviors (Calhoon and Tye 2015). The use of modern methodologies such as optogenetics, among others, in rodents has led to the improved and more

detailed characterization of the intricate interplay between the different brain loci forming circuits to mediate and modulate anxiety-like behavior (Tovote et al. 2015; Orsini and Maren 2012; Dias et al. 2013; Calhoon and Tye 2015). At the center of this circuitry is the extended amygdala, important for the emotional interpretation of environmental information as it arises, determining whether downstream fear (see below) or reward pathways (e.g., via the nucleus accumbens, NA) will be activated. The extended amygdala includes different amygdala subregions, including the basolateral amygdala (BLA) and the central amygdala (CeA; composed of the centro-lateral (CeL) and centro-medial (CeM) amygdala), as well as the bed nucleus of the stria terminalis (BNST), which coordinates inputs from the amygdala and hippocampus. Together with other regions, such as the hypothalamus (HYP), they also modulate the neuroendocrine stress response. Other brain regions implicated in the modulation of anxiety-related behaviors include the medial prefrontal cortex (mPFC); the highly interconnected hippocampus, which encodes contextual aspects important for anxiety, and important modulatory monoaminergic cell body areas; the locus coeruleus (LC); the raphe nucleus (RN); and the ventral tegmental area (VTA) (Fig. 1, top panel) (for detailed review, see Tovote et al. 2015). The signal processing between these brain areas and neuronal populations is driven by a finely tuned interplay of different neurotransmitters and neuromodulators, including neurotransmitters such as gamma-aminobutyric acid (GABA); glutamate; monoamines such as serotonin, norepinephrine, and dopamine; and a number of neuropeptides, including neuropeptide Y (NPY), neuropeptide S (NPS), endocannabinoids, cholecystokinin (CCK), and calcitonin gene-related peptide (CGRP), which have been associated with anxiety regulation and considered as drug targets (Bowers et al. 2012; Wang and Pereira 2016; Millan 2003). Many anxiety disorders are considered stress-related, and thus regulation of the corticotropin-releasing factor (CRH) system and of the hypothalamic-pituitary-adrenal (HPA) axis has been found to be important players, particularly in stress-induced anxiety (Shin and Liberzon 2010; Bartlett et al. 2017). Aberrant anxiety reactions are thought to arise from altered processing and deficiencies in such circuitries, including imbalances in neurotransmitter regulation and downstream signaling pathways (Calhoon and Tye 2015; Millan 2003). Mechanisms that can orchestrate and restore this delicate balance are sought in the quest for more rational therapeutic targets in anxiety treatment. MicroRNAs are such candidates (see below), having been shown to interact with such regulatory neurotransmitters and signaling cascades (see below and Martinetz 2016, for review).

The mechanisms leading to deficiencies in the networks processing anxiety are not understood in detail. Family and twin studies have indicated that anxiety disorders are multifactorial in cause, involving an interaction between genetic and environmental factors (Hettema et al. 2001, 2005; Klengel and Binder 2015; Sharma et al. 2016). Heritability in anxiety disorders is rather moderate but can amount to 30–48%, for example, in generalized anxiety/panic disorder (Hettema et al. 2001; Kendler et al. 1999; Shimada-Sugimoto et al. 2015). Evidence suggests that multiple genes with small effects are involved, with single gene variation insufficient to produce full anxiety pathology. Genome-wide association studies

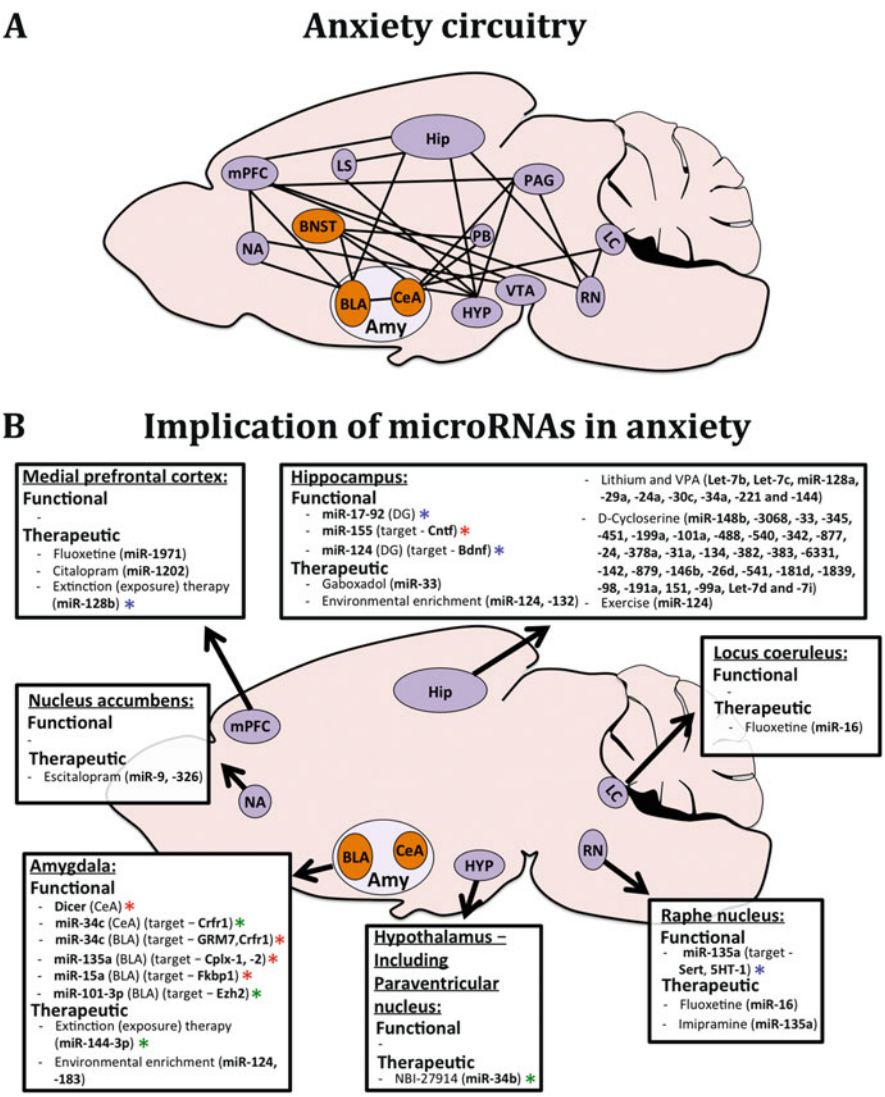

Fig. 1 Brain areas investigated for a (functional) role of microRNAs in anxiety. (**a**) Schematic of proposed anxiety circuitry based on references (Calhoon and Tye 2015; Tovote et al. 2015). (**b**) Schematic representation summarizes the most recent studies assessing microRNAs in the brain for regional, functional, and therapeutic implications in anxiety-related behaviors. Studies that assessed the implications of candidate microRNA expression using functional approaches are indicated with an asterisk (∗), while red (knockdown), green (overexpression/enhancement), and blue (both) indicate the direction of regulation. Bed nucleus of the striatum terminalis (BNST), medial prefrontal cortex (mPFC), hippocampus (Hip), amygdala (Amy; amygdala subregions: basolateral amygdala (BLA) and central amygdala (CeA)), nucleus accumbens (NA), raphe nucleus (RN), hypothalamus (HYP), locus coeruleus (LC), periaqueductal gray (PAG), parabrachial nucleus (PB), ventral tegmental area (VTA), and lateral septum (LS)

(GWAS) assessing the presence of single nucleotide polymorphisms (SNPs) have revealed a number of chromosomal risk loci, including genes associated with neurotransmitter and HPA axis regulation, such as serotonin receptor 1A (5HT-1A), catechol-O-methyltransferase (COMT), monoamine oxidase A (MAOA) genes, neuropeptide S receptor (NPSR), brain-derived neurotrophic factor (BDNF), and HPA axis genes corticotropin-releasing hormone receptor 1 (CRHR1) and FK506 binding protein 5 (FKBP5) (Smoller 2016; Bandelow et al. 2016; Domschke et al. 2012; Autry and Monteggia 2012).

The remaining variability associated with the development of anxiety-related disorders is accounted for predominantly by environmental factors (Hettema et al. 2005; Vieland et al. 1996; Norrholm and Ressler 2009; Schiele and Domschke 2017). Stress is particularly well studied in this regard (Dirven et al. 2017). For example, stress during pregnancy, neonatal stress, or traumatic experiences during childhood, such as parental loss or physical/emotional neglect, affect the function of various neurobiological mechanisms relevant for anxiety processing, including the HPA axis (Maccari et al. 2014), and are factors that have been linked with increased vulnerability to the development of anxiety disorders later in life (Stein et al. 2014; Drury et al. 2016; Fernandes and Osorio 2015; Klauke et al. 2010). Research suggests changes in gene expression of multiple known candidate genes such as genes involved in DNA transcription and translation play a role. The discovery in recent years of epigenetic mechanisms and their role in the control of gene expression made important contributions to our understanding of the complex development of anxiety-related disorders. It is thought that these mechanisms may explain how the combination of an accumulation of affected risk genes and environmental insults (G X E interaction) lead to the onset of anxiety-related disorders (Sharma et al. 2016) (Fig. 2).

3 Epigenetics in Anxiety: MicroRNAs

"Epigenetics" is the term given to mechanisms that together control gene expression in the absence of any alterations to the DNA base pair composition (Bjornsson et al. 2004). Epigenetic marks involved in the modulation of gene expression can be influenced by environmental factors, can be inherited to influence subsequent generations, but, importantly, can also be reversed (Jaenisch and Bird 2003), suggesting a dynamic modulation mediated by these marks. These mechanisms exert multiple levels of control over gene expression, ranging from DNA and histone modifications to posttranscriptional regulation via noncoding RNAs, including microRNAs (Dias et al. 2015; Cholewa-Waclaw et al. 2016), and they have emerged as critical regulators in nearly all neurobiological processes (Sevignani et al. 2006; Ong and Corces 2011). Environmental insults, such as stress, diet, or toxin exposure, can affect epigenetic mechanisms, which could explain their profound and long-lasting effects on multiple neurobiological systems and networks. Epigenetically mediated mechanisms could also shed light on individual variations

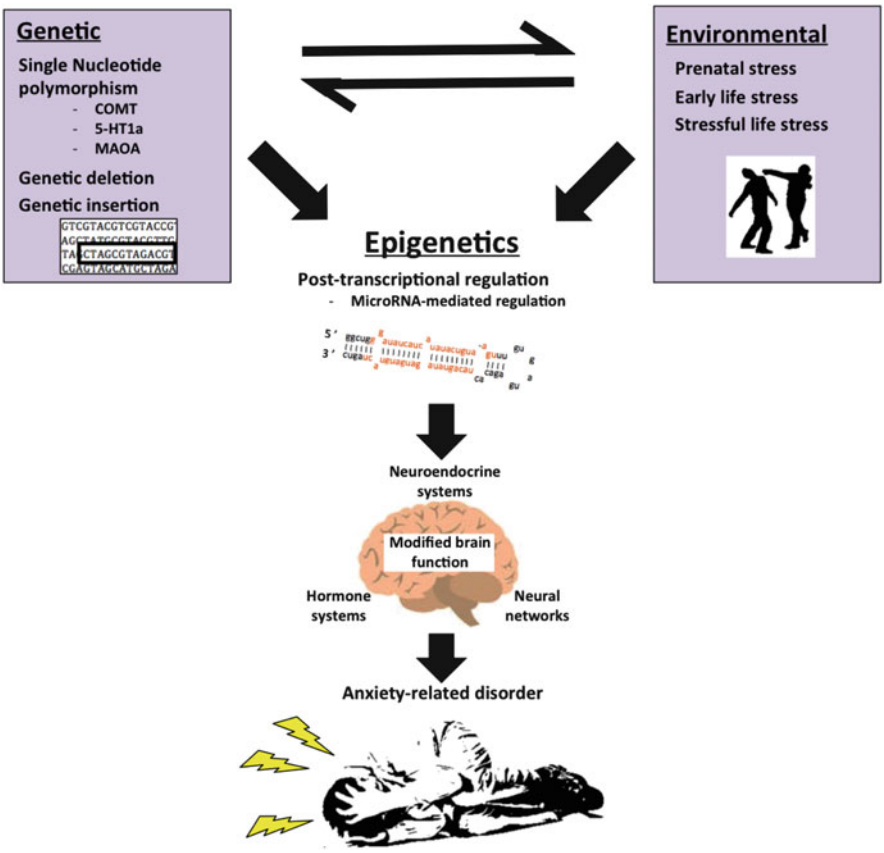

Fig. 2 Genetic/environmental/epigenetic influences in anxiety-related disorders. Schematic representation of gene × environment × epigenetics in the development of anxiety-related disorders. Genetic and environmental factors can lead to long-lasting changes in epigenetic mechanisms, resulting in changes in brain structure and also functional brain processing. These changes can subsequently lead to the development of anxiety-related disorders

in the development and treatment of some of the most common central nervous system (CNS) disorders (Yehuda and Bierer 2009; Landgrave-Gomez et al. 2015), including anxiety disorders (Nieto et al. 2016; O'Connor et al. 2016; Bartlett et al. 2017). Environmental influences associated with the development of anxiety disorders have been linked with distinct changes in epigenetic mechanisms, such as histone acetylation, DNA methylation, and noncoding RNA expression, which regulate the long-lasting expression of specific genes (Schiele and Domschke 2017). Genes targeted are, for example, associated with neurotransmission/neuromodulation, including GABAergic, glutamatergic, dopaminergic and serotonergic signaling, or genes associated with HPA axis functioning, many of them known to be aberrantly regulated in anxiety (Fass et al. 2014; Hunter 2012).

MicroRNAs are one of the best-studied posttranscriptional modifiers of gene expression, revealing their critical role in CNS development and homeostasis. As such, they have emerged as interesting candidates for studying molecular mechanisms underlying anxiety and anxiety disorders (Scott et al. 2015). Furthermore, the standardization of methods of examining microRNA-mediated effects (Baker 2010) and the recent increase in functional studies assessing causal roles of microRNAs have generated an opportunity to reassess where we stand with regard to microRNA-mediated mechanisms and anxiety. In the next sections, we briefly summarize microRNA function in the brain and then focus on an update on the current status of the role microRNAs are thought to play in the development, progression, and treatment of anxiety-related disorders.

3.1 MicroRNAs in the Central Nervous System

MicroRNAs are short (19–24-nucleotide) single-stranded ncRNAs that are genomically encoded and that mediate the posttranscriptional regulation of target genes via complementary binding to the messenger RNA seed sequence (Bartel 2009). There have been over 5,000 microRNA transcripts annotated in humans so far, together comprising 1–2% of the human genome (Londin et al. 2015). It is well documented that microRNAs can target hundreds of genes often within distinct functional networks via partial complementary binding and, furthermore, any given gene may have multiple target sites for different microRNAs (Lim et al. 2005; Wang et al. 2011). Originally thought of as "junk RNA", such as leftover remnants of past viral infections, it has now been revealed that microRNAs may control the expression of up to 60% of the protein-coding genes in the human genome in a highly complex network-like fashion (Krol et al. 2010b; Bartel 2004). The first microRNA to be discovered was lin-4, which was found in *Caenorhabditis elegans* using a genetic screening tool for gene variations associated with deficits in the temporal control of postembryonic development (Lee et al. 1993). In 2001 the true potential of these small noncoding RNAs was revealed when their role as regulators of gene expression was demonstrated (Lau et al. 2001). Since this initial breakthrough discovery, the emergence of microRNAs and their downstream effects on target genes has revealed them as "fine tuners" of gene expression, and this has subsequently become one of the best-studied mechanisms of posttranscriptional regulation.

3.2 MicroRNA Induction, Biogenesis, Turnover, and Function

Induction, biogenesis, turnover, and general function of microRNAs have been the subject of specific reviews; for a detailed discussion on this topic, please see Ruegger

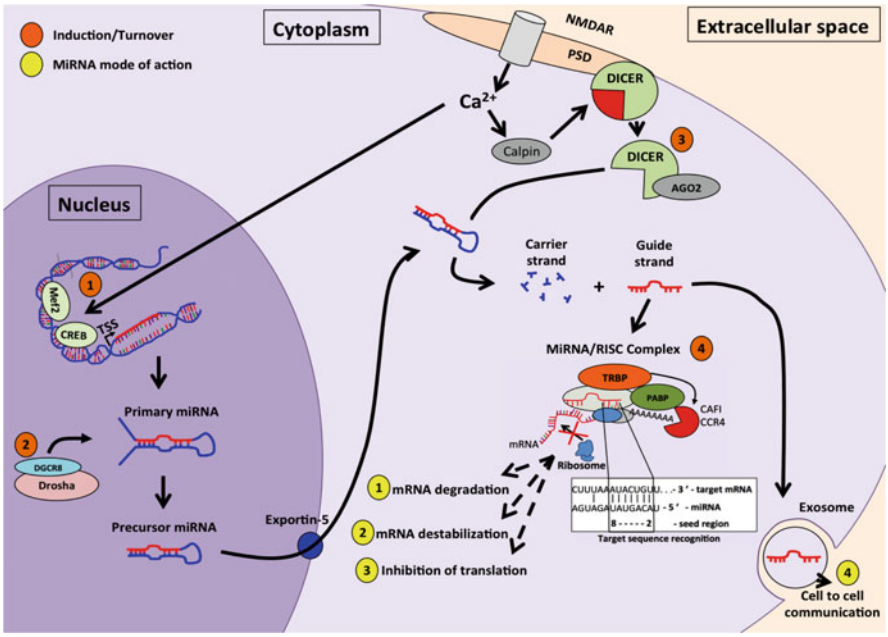

Fig. 3 MicroRNA induction, biogenesis, turnover, and function. MicroRNA expression is induced in a number of ways including via neuronal activation whereby (1: orange) Ca^{2+} influx influences the expression of transcriptions factors such as MEF2 and CREB, which subsequently have been shown to mediate the expression of a wide range of microRNAs. Following transcriptional induction, RNA polymerase II transcribes microRNAs as precursor microRNAs (pri-miRNA) from specific microRNA genes or from within the introns of protein-coding genes. These pri-miRNAs are then processed by a member of the RNase III enzyme family, Drosha, and along with other cofactors, including DiGeorge syndrome critical region gene 8 protein (DGCR8), cleave the pri-miRNA into a 70–100-nucleotide-long pre-miRNA. (2: orange) microRNA turnover is in part controlled by the regulation of Drosha where decreased expression reduces the ability of a cell to transcribe new microRNAs. Exportin-5 then transports the pre-miRNA to the cell cytoplasm where Dicer processes it into a microRNA duplex. (3: orange) Ca^{2+} influx is also associated with increased Calpain expression, which releases Dicer from the postsynaptic densities (PSD). Along with Argonaute 2 (Ago2) and transactivation-responsive RNA-binding protein (TRBP), Dicer incorporates the microRNA "guide" strand, based on the thermodynamic asymmetry rule, into the microRNA-induced silencing complex (miRISC). The co-processed "passenger" strand is degraded at this point. The microRNA and miRISC complex then induce (1 and 2: yellow) mRNA degradation/destabilization and/or (3: yellow) translational repression via target gene binding through a "seed sequence" located between nucleotides 2 and 8 on the microRNA guide strand (enclosed panel). PABP, poly(A)-binding protein. CCR4, C-C motif chemokine receptor 4

and Grosshans (2012), Vidigal and Ventura (2015), Krol et al. (2010b), Bartel (2004, 2009), and Sim et al. (2014). Here, we will give a brief account of these processes (*summarized in* Fig. 3).

In the nervous system, the induction of microRNA expression can occur via a number of different mechanisms of which the most studied is via neuronal activity (Sim et al. 2014). Generally speaking, Ca^{2+} influx following neuronal activation

can lead to various downstream effects including the induced expression of transcription factors such as MEF2 and BDNF, which have subsequently been shown to mediate the expression of specific microRNAs such as miR-132 (Nudelman et al. 2010). Following the induction, precursor microRNA molecules (pri-miRNAs) are transcribed by RNA polymerase II, with approximately half of the microRNA genes being transcribed from their own nonprotein-coding transcripts and the other half from within the intergenic regions of protein-coding genes (Saini et al. 2007). Pri-miRNA transcripts are then subject to processing whereby they fold into hairpin structures that are subsequently bound by a member of the RNase III enzyme family, Drosha. Drosha itself is subject to modulation, which is another rate-limiting step on the biogenesis of mature microRNAs. Drosha, with cofactors including DiGeorge syndrome critical region gene 8 protein (DGCR8), cleaves the pri-miRNA into a 70–100-nucleotide-long pre-miRNA, which is subsequently exported to the cell cytoplasm by exportin-5. In the cytoplasm, another RNase III family enzyme, Dicer, removes the hairpin structure and processes the pre-miRNA into a microRNA duplex. In combination with the RNA-binding proteins, Argonaute 2 (Ago2) and the transactivation-responsive RNA-binding protein (TRBP), Dicer, incorporate the microRNA guide strand, based on the thermodynamic asymmetry rule, into the microRNA-induced silencing complex (miRISC), while the other "passenger" strand is degraded. The thermodynamic asymmetry rule implies that the microRNA strand with the least stable 5′ terminus will be chosen as the "guide" strand and incorporated into the miRISC complex, while the other "passenger" strand is degraded. Any given microRNA has a 5p and a 3p, which is dependent on what side of the hairpin each microRNA strand is situated. In principle, both the 5p and the 3p strand can act as the guide strand. Once incorporated into the miRISC complex, microRNAs then induce translational repression and/or mRNA degradation via binding with target genes through a "seed sequence" located between nucleotides 2 and 8 on the microRNA guide strand (Fig. 3, enclosed panel). A microRNA whose seed sequence has perfect complementarity with its target gene will have the greatest effect on the expression levels of this target.

While some microRNAs are expressed ubiquitously, such as let-7b, miR-17, and miR21, there are a number of microRNAs that are enriched in the brain, such as miR-128b (Lin et al. 2011), as well as microRNAs that are expressed in a temporal-, regional-, and cell-type-specific manner (Taguchi 2013), as shown for miR-7, miR-34a, and miR-132, among others, in the brain (Olsen et al. 2009). MicroRNA-mediated control over mechanisms such as gene expression regulation in the nervous system makes them essential mediators of important processes in the brain, including neuronal plasticity and learning and memory (Ruegger and Grosshans 2012). MicroRNA expression/turnover within neurons can be relatively fast. In the mouse retina, for example, light-sensitive microRNAs such as miR-204 and miR-211 and the microRNA-cluster miR-183/96/182 undergo rapid transcriptional repression, e.g., 30 min following adaption of the mouse to the dark chamber (Krol et al. 2010a). This microRNA regulation has subsequently been found to play a functional role, preventing retinal degeneration (Lumayag et al. 2013). A number of studies have further characterized microRNA turnover in the brain and have

shown that microRNA expression is dynamic and under the control of a number of different mechanisms, including those involved in biogenesis, maturation, and degradation (Marzi et al. 2016) (Fig. 3). For example, one approach blocking the biogenesis of all new microRNAs, via ablation of Dicer, led to the discovery of varying half-lives of investigated microRNAs, ranging from 5 to 12 days (Gantier et al. 2011). Thus, microRNAs in general can be induced within minutes (e.g., by neuronal activity in neurons (Sim et al. 2014)) and can remain within cells in a stable condition for at least several days and maybe much longer (Gantier et al. 2011).

3.3 *MicroRNAs as Modulators of Neuronal Plasticity and Learning and Memory*

Adaptive responses to environmental stimuli are mediated via neuronal activity at a cellular level and involve a number of molecular changes altering synaptic dynamics, including local synaptic expression of plasticity-associated proteins. This expression is controlled by a number of synaptically located microRNAs, induced by neuronal activity, which ensures fine-tuned control over synaptic plasticity (Lugli et al. 2008; Hu et al. 2014; Schratt 2009; Gu et al. 2015) and learning and memory mechanisms (Wang et al. 2012). For example, miR-132 has emerged as an interesting candidate. High-frequency stimulation-induced long-term potentiation in the dentate gyrus reflecting enhanced synaptic plasticity is associated with increased expression levels of miR-132 (Wibrand et al. 2010). It was suggested that miR-132, depending on brain region and/or behavioral-specific signals, can act as a "proteome switch" and mediate different mechanisms of neuronal plasticity through its target genes, including the N-methyl-D-aspartate receptor subunit 2A (Bredy et al. 2011). The critical role played by microRNAs in these processes, which include learning and memory and related disorders, has been the topic of detailed reviews (please see Saab and Mansuy 2014; Hu and Li 2017; Aksoy-Aksel et al. 2014).

In relation to anxiety disorders, it should be mentioned that disturbances in learning and memory mechanisms are common in these disorders (Hemstedt et al. 2017), which are increasingly targeted by specific therapeutic interventions (e.g., Singewald et al. 2015). There is mounting evidence linking microRNAs to these processes (see below).

3.4 *MicroRNAs in Anxiety and Anxiety-Related Disorders: Associative Studies*

There is increasing evidence of microRNA dysregulation in disease. By far the best-studied field so far is cancer (for more detailed reviews, please see Kong et al. 2012; Nana-Sinkam and Croce 2013; Garzon et al. 2009). Concerning the brain,

dysregulation of microRNAs has also been demonstrated in brain tumors, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, and, more recently, psychiatric disorders (Spadaro and Bredy 2012; Wang et al. 2012; You et al. 2016; Cao et al. 2016; O'Connor et al. 2016). In particular, the investigation of microRNAs in psychiatric disorders has gained momentum due to their potential application as molecular biomarkers and/or their use in microRNA-specific therapies for psychiatric illness (Kichukova et al. 2015; Scott et al. 2015).

Because of the inaccessibility of brain tissues in living patients, human studies investigating the role of microRNAs in psychiatric patients rely on postmortem brain tissues obtained from patients with higher mortality rates due to higher risk-taking and/or higher suicide rates accompanying disorders such as bipolar disorder, schizophrenia, and major depressive disorder (Rao et al. 2013). Little is known regarding brain microRNA changes in anxiety disorders on their own, but there are studies in comorbid patients suffering from anxiety disorders and depression showing altered microRNA regulation (see below).

The majority of human studies examining anxiety-related disorders are limited to the assessment of differential microRNA expression in the periphery – mostly in blood and its constituents – and the correlation of these findings with the presence of disease (Table 1). For example, in generalized anxiety disorder (GAD) patients, circulating miR-663 and miR-4505 levels correlated with anxiety symptoms (Chen et al. 2016), and miR-29c expression increased in response to social anxiety in a social stress task, corresponding both to the stress experience and to alterations in ventromedial prefrontal cortex functional connectivity (Vaisvaser et al. 2016). Other insights came from postmortem samples of patients who had suffered from depression comorbid with anxiety, which revealed a downregulation of miR-135a in the raphe nucleus (Issler et al. 2014). Introducing further complexity, single nucleotide polymorphisms (SNPs) in the precursors or mature microRNAs could interfere with balanced processing in anxiety (for review see Malan-Muller et al. 2013). For example, the search for SNPs associated with specific microRNA candidates revealed a number of microRNA variations, including miR-22, miR-138-2, miR-148a, miR-339, and miR-488, which are associated with anxiety-related disorders such as panic disorder (Muinos-Gimeno et al. 2011). Some microRNA candidates with SNPs, such as miR-22, were also identified in a different cohort of panic disorder patients (Kim et al. 2015). As another example, polymorphisms in the Dicer1 gene have been linked with PTSD (Wingo et al. 2015). These studies demonstrated aberrant microRNA expression and possibly regulation in anxiety and provided important indication of a role of microRNAs in anxiety-related disorders. For more detailed discussion of these findings, please see recent reviews (Snijders et al. 2018; Martinetz 2016; Scott et al. 2015; Issler and Chen 2015; Malan-Muller et al. 2013; O'Connor et al. 2012, 2016; Hommers et al. 2015). These correlative studies are very important but have the disadvantage that it is hard to conclude whether microRNA expression and its subsequent posttranscriptional regulation causes the anxiety disorder or is a consequence of it. This is where the use of relevant animal models of anxiety is invaluable. Indeed, there are preclinical studies emerging that attempt to provide causal evidence for a functional role of microRNAs in anxiety. In the next sections, we focus primarily on these studies.

Table 1 Altered microRNA levels in human anxiety and anxiety-related disorders

MicroRNA(s)	Disorder	Tissue	Trial design	Results	Ref
miR-570, miR-219, miR-637, miR-668, miR-519a, miR-518f, miR-615, miR-125a, and miR-181c	PTSD	PBMC	30 PTSD vs 42 HC	miR-570, miR-219, miR-637, miR-668, miR-519a, miR-518f, and miR-615 expression is increased, while miR-125a and miR-181c expression is decreased in PTSD patients	Zhou et al. (2014)
miR-144 and miR-16	Anticipatory anxiety	Blood plasma	Ten healthy medical students	miR-144 and miR-16 expression in blood plasma increased in the run-up to a stressful exam, peaking on the day of the exam before returning to baseline expression following	Katsuura et al. (2012)
miR-144	Depression comorbid with anxiety	Blood plasma	169 patients vs 52 HC	miR-144 expression is lower in patients with depression and anxiety. Following group therapy, the expression levels of miR-144 increased	Wang et al. (2015)
miR-3130	PTSD/ depression	Blood	34 PTSD/ depression vs 20 healthy controls	miR-3130 is decreased in patients who suffer from PTSD/ depression	Wingo et al. (2015)
miR-29c	Social stress	PBMC	49 HC	Social stress was associated with increased expression of miR-29c in PBMCs, which furthermore correlated with increased connectivity of the vmPFC with the anterior insula and decreased connectivity with the left dorsolateral PFC	Vaisvaser et al. (2016)
miR-4484, miR-4674, miR-501, miR-663,	Generalized anxiety disorder	PBMC	76 patients vs 39 HC	miR-4484, miR-4674, miR-501, miR-663,	Chen et al. (2016)

(continued)

Table 1 (continued)

MicroRNA(s)	Disorder	Tissue	Trial design	Results	Ref
miR-4505, miR-1301, and miR-432				and miR-4505 expression was elevated in PBMCs of patients with generalized anxiety, while the expression levels of miR-1301 and miR-432 were decreased	
miR-15a	PTSD/ depression	Whole blood	Male individuals	miR-15a expression was increased in the peripheral blood following DEX administration or childhood trauma exposure	Volk et al. (2016)
miR-193a	PTSD (Male combat veterans)	PBMC	16 PTSD vs 17 HC	Expression levels of miR-193a are decreased in the PBMCs of combat veterans suffering from PTSD	Bam et al. (2016a)
Differential regulation of 190 microRNAs	PTSD (male combat veterans)	PBMC	24 PTSD vs 24 HC	In male combat veterans suffering from PTSD, 183 microRNAs exhibit decreased expression, while 7 microRNAs display enhanced expression in PBMCs	Bam et al. (2016b)
miR-19a, miR-101, miR-20b, miR-20a, miR-486, miR-128, miR-15b, and miR-125b	PTSD (Male combat veterans)	Whole blood	15 PTSD vs 9 HC	The expression of miR-19a, miR-101, miR-20b, and miR-20a are increased while miR-486, miR-128, miR-15b, and miR-125b exhibit decreased expression in the blood of combat veterans who suffer from PTSD	Martin et al. (2017)

3.5 *MicroRNAs in Anxiety and Anxiety-Related Disorders: Studies Elucidating Functional/Causal Evidence*

As mentioned above, microRNAs are abundant in the brain, where they appear to be differentially distributed, including the anxiety circuitry (Fig. 1), and even differently within neurons and distinct neuronal compartments. Functional implications of microRNAs in anxiety have been revealed using animal tests and models showing that down- or up-regulation of the candidate microRNAs in important nodes of the anxiety circuitry can indeed modulate anxiety-related behavior. The results of these studies are discussed below and are summarized in Table 2. In Fig. 1 (bottom panel), an overview is given of where in the brain such functional relationships have been investigated so far.

The first to explore the role of microRNAs in anxiety with a more functional approach were Haramati and colleagues, who locally depleted the microRNA-processing enzyme, Dicer, in the central amygdala of adult mice. As predicted, this depletion led to a drastically reduced ability for central amygdalar neurons to generate new mature microRNAs, which was found to be associated with increased anxiety-like behavior in the light/dark and open-field tests (Haramati et al. 2011). Since stress, as mentioned, is a major contributor to the development and triggering of anxiety-related behavior, stress-associated microRNA regulation has been particularly studied as a means of bridging stress with downstream pathology (Issler and Chen 2017). For example, in investigating mice following acute and chronic stress, miR-34c was revealed as a prominent stress-induced microRNA in the central amygdala. The authors suggest this microRNA plays a role in stress resilience and the development of anxiety. It was revealed that its upregulation reduced anxiety-like behavior via downregulating the expression of its target gene, corticotropin-releasing hormone receptor 1 (CRHR1), a key player in stress-induced anxiety. Furthermore, miR-34c expression was decreased in the central amygdala of Dicer-ablated neurons, which was associated with increased anxiety-like behavior (Haramati et al. 2011). Together these data highlight miR-34c expression in the central amygdala as a prominent microRNA involved in resilience to anxiety following stress. Attempting to further characterize the functional role of this microRNA, overexpression of miR-34c in the central amygdala produced an anxiolytic effect, demonstrating that manipulation of a single microRNA in one important node of the anxiety circuitry can drive a behavioral response (Haramati et al. 2011). Somewhat in contrast to these findings, Andolina and colleagues demonstrated that knockout (rather than overexpression) of miR-34 in the BLA is associated with resilience against stress-induced anxiety and with the facilitation of fear extinction (Andolina et al. 2016). Discrepancies in miR-34-mediated effects on anxiety behavior may be attributed to a number of reasons, including the fact that two different amygdalar subnuclei were investigated. Haramati et al. performed a lenti-virally mediated overexpression of miR-34c in the central amygdala, whereas Andolina et al. knocked out miR-34 in the BLA. This could suggest distinct regionally specific roles played by specific candidate microRNAs such as miR-34

Table 2 MicroRNAs functionally involved in anxiety-related behaviors

MicroRNA(s)	Species	Test	Region	Effect	Gene target(s)	Refs
Functional (anxiety paradigms)						
Dicer	Mouse	*Anxiety-like behavior*: Light-dark test, Open field test	Central amygdala	*CeA Dicer depletion* led to *increased anxiety-like behavior*	–	Haramati et al. (2011)
miR-15a	Mouse	*Stress-induced anxiety*: Elevated plus maze	Amygdala (basolateral amygdala)	Mice exposed to *chronic stress* were associated with increased *amygdalar miR-15a* expression. miR-15a *knockdown* in the *basolateral amygdala* led to *increased anxiety-like behavior* in mice, suggesting that miR-15a expression is important for *stress adaption*	Fkbp1	Volk et al. (2016)
miR-17-92	Mouse	*Anxiety- and depressive-like behavior*: Elevated plus maze, open field test, forced swim test, tail suspension test	Hippocampus (dentate gyrus)	*miR-17-92 depletion* in adult *neural progenitor* cells was associated with *decreased neurogenesis* in the *dentate gyrus* whereas *overexpression* led to *increased neurogenesis*. Furthermore, miR-17-92 *knockout* mice exhibit *increased anxiety*- and *depressive*-like behaviors while *overexpression* mice display *anxiolytic* and *antidepressive*-like behavior	–	Jin et al. (2016)
mir-34c	Mouse	*Stress-induced anxiety*: Light-dark test, elevated plus maze	Central amygdala	*Overexpression* of *miR-34c* led to the *protection* against stress-induced *anxiety*	Crfr1	Haramati et al. (2011)
miR-34	Mouse	*Stress-induced anxiety*: Light-dark test, elevated plus maze, open field test, fear extinction	Amygdala (basolateral amygdala)	*miR-34 knockout* was associated with *increased resilience* to *stress-induced anxiety*. Furthermore miR-34 knockout *facilitated fear extinction*	Grm7, 5HT-2c, Crfr1	Andolina et al. (2016)
miR-124a	Rats	*Neonatal isolation-induced anxiety*: Open field test, elevated plus maze	Hippocampus (dentate gyrus)	*miR-124a expression* is *increased* in the *dentate gyrus* following *neonatal isolation*, which is associated with *increased anxiety-like behavior*. Furthermore, *overexpression* of miR-124a in the *dentate gyrus* further *exacerbated anxiety-like behavior*	Bdnf	Bahi (2016)

miR-124a	Rats	*Neonatal isolation-induced anxiety*: Open field test, elevated plus maze	Hippocampus (dentate gyrus)	Virally mediated *decreased miR-124a expression* in the *dentate gyrus* was associated with *decreased anxiety-like behavior*	Bdnf	Bahi (2017)
miR-101-3p	Rats	*Trait-anxiety*: Open field test, elevated plus maze	Amygdala (basolateral amygdala)	Rats selectively bred for *high novelty responding* (low trait anxiety) exhibit lower expression levels of miR-101a-3p in the *amygdala, compared* to rats bred for *low novelty responding* (high trait anxiety). *Virally enhancing miR-101-3p* expression in *high novelty responding rats* led to *enhanced anxiety-like behavior* in open-field test and elevated-plus maze	Ezh2	Cohen et al. (2017)
miR-135a	Mouse	*Anxiety- and depressive-like behavior*: Social interaction following social defeat, elevated plus maze, light-dark test	Raphe nucleus	*Overexpression* of *miR-135a* in the *raphe nucleus* led to reduction in *anxiety* and *depressive-like behavior* following *social defeat*. Whereas *knock-down* of *miR-135a* led to *increased anxiety-like behavior*	Sert, 5HT-1a	Issler et al. (2014)
miR-135a	Mouse	*Anxiety-like behavior*: Elevated plus maze	Amygdala (basolateral amygdala)	*Knockdown* of *miR-135a* in the *basolateral amygdala* was associated with *increased anxiety-like behavior* and furthermore was shown to mediate *glutaminergic neurotransmission*	Cplx-1 and -2	Mannironi et al. (2017)
miR-155	Mouse	*Anxiety- and depressive-like behavior*: Elevated plus maze, open field test, forced swim test, tail suspension test	Hippocampus	*miR-155 depletion* was associated with *reduced anxiety-* and *depressive-like* behavior	Cntf	Fonken et al. (2016)

in the central amygdala vs the BLA. Further underlining the importance of this microRNA, members of the miR-34 precursor family have emerged as critical regulators in a number of amygdalar-dependent learning paradigms, including fear learning. For example, it has been demonstrated that miR-34a expression is increased in the amygdala 30 min following auditory fear conditioning, while knockdown of this microRNA in the basolateral amygdala impairs fear learning (Dias et al. 2014). These data suggest that the miR-34 precursor family plays a critical role in amygdalar-dependent memory formation, with dissociated effects on fear and extinction learning, whereas knockdown of miR-34 in the basolateral amygdala can facilitate fear extinction and at the same time impairs the formation of fear memory.

As discussed above, the RDoC initiative is fostering trans-diagnostic research investigating endophenotypes, to evaluate neurobiological mechanisms that may cut across traditional diagnoses. In line with the high comorbidity in anxiety disorders, involving multiple anxiety disorder categories and other comorbid conditions such as mood disorders, a number of studies assessing the functional role of microRNAs in animal models have investigated both anxiety- and depressive-like behaviors. Fonken and colleagues demonstrated that a genetic knockout of miR-155 in the hippocampus was associated with reduced anxiety- and depressive-like behavior (Fonken et al. 2016). Using a similar methodology, knockout of miR-17-92 in adult neural progenitor cells led to decreased neurogenesis in the dentate gyrus of the hippocampus and increased anxiety- and depressive-like behavior, while overexpression produced the opposite effects on neurogenesis and behavior (Jin et al. 2016). This is in line with reports linking enhanced trait anxiety with reduced hippocampal neurogenesis (Sah et al. 2012). The functional relationship between anxiety-related behavior and microRNA-mediated modulation has so far been demonstrated in three different brain areas in the anxiety circuitry: the amygdala, the hippocampus, and the raphe nucleus (Fig. 1, bottom panel). For example, overexpression of miR-135a in the raphe nucleus led to a reduction in anxiety- and depressive-like behavior elicited by social defeat, while miR-135a knockdown was associated with increased anxiety-like behavior (Issler et al. 2014). The authors found these effects to be mediated via interaction of miR-135a with the serotonergic system, targeting specifically the serotonin transporter (SERT) and serotonin receptor 1a (5HT-1a) genes in the dorsal raphe nucleus. As stated in the previous sections, microRNAs can target numerous genes and have varying effects in different cell types and tissues/brain areas due to distinct expression patterns and induction pathways. In agreement with this, a recent study demonstrated that knockdown of miR-135a in the basolateral amygdala also increased anxiety-like behavior, but in this brain area, it was found that miR-135a regulates anxiety via interaction with glutamatergic neurotransmission, targeting in particular complexin-2 (Mannironi et al. 2017).

Investigating microRNAs in trait anxiety in rats, Cohen and colleagues found that rats selectively bred for high novelty responding – which is associated with low trait anxiety – exhibit lower expression of miR-101a-3p in the amygdala compared with rats bred for low novelty responding that show high trait anxiety (Cohen et al. 2017).

Supporting the relation of miR-101-3p regulation to anxiety-like behavior, overexpression of miR-101-3p in the basolateral amygdala was indeed associated with enhanced anxiety-like behavior. In this relation it should be mentioned that recent results suggest that the gut microbiome is necessary for appropriate regulation of microRNA expression in brain regions implicated in anxiety-like behaviors including the amygdala and prefrontal cortex, adding an additional level of complexity (Hoban et al. 2017). Also in hippocampal areas, functional microRNA regulation was investigated. Using a model of neonatal isolation-induced anxiety, Bahi revealed that expression of the brain-enriched miR-124a was increased in the dentate gyrus of neonatally isolated Wistar rats (Bahi 2016). Overexpression of miR-124a in the dentate gyrus further exacerbated the anxiety-like behavior, demonstrating that the intensity of the anxiety response correlates with the expression level of miR-124a in the dentate gyrus. Supporting these findings, the author more recently demonstrated that virally mediated reduction of miR-124a expression in the dentate gyrus is associated with decreased anxiety-like behavior, which inversely correlates with the expression of its target gene, brain-derived neurotrophic factor (BDNF), in this brain area (Bahi 2017). BDNF is an activity-dependent neurotrophic factor that has been extensively implicated in fear and anxiety regulation (Andero et al. 2014; Andero and Ressler 2012). The increase in functional evidence linking microRNA regulation with anxiety supports a crucial role played by these "fine tuners" of gene expression and highlights them as possible novel therapeutic targets for the treatment of anxiety disorders.

3.6 MicroRNAs in the Treatment of Anxiety-Related Disorders

MicroRNAs have been investigated as therapeutic targets for numerous disorders, including in particular the treatment of cancer, with the first cancer-targeted microRNA, MRX34, entering clinical trials in patients with advanced hepatocellular carcinoma (Ceman and Saugstad 2011; Kong et al. 2012; Ling et al. 2013). Miravirsen, which targets miR-122, was recently demonstrated in a phase II trial to have no long-term safety issues and sustained its virological response, which is associated with reduced occurrence of liver failure, in the treatment of chronic hepatitis C (van der Ree et al. 2014). More recently, microRNAs have also been studied as potential targets in psychiatric disorders (Tardito et al. 2013; O'Connor et al. 2012, 2016). One idea here is that microRNA-mediated regulation of relevant biological processes is linked with the therapeutic effect of anxiety disorder treatments. As studies functionally assessing microRNAs in the therapeutic action of drugs are scarce, here we have also included correlative studies that specifically focused on drug-induced changes in microRNA expression associated with a therapeutic decrease in anxiety-like behavior.

3.7 *MicroRNA Regulation in Established and Experimental Pharmacological Interventions Used in Anxiety Treatment*

A number of studies have assessed the possible contribution of specific microRNAs to the therapeutic action of currently used interventions for anxiety disorders (summarized Fig. 1 (bottom panel)). Indeed, medications used in anxiety and affective disorders have been shown to modulate microRNA levels in the brain, although microRNAs were not their original target. One of the first of such studies demonstrated that long-term administration of different mood stabilizers led to altered expression of microRNAs in the hippocampus of rats when compared with saline-treated controls (Zhou et al. 2009). Following chronic oral treatment with valproate (VPA) or lithium, a number of microRNAs, including miR-29a, Let-7b, Let-7c, miR-128a, miR-24a, miR-30c, miR-34a, and miR-221, exhibited decreased hippocampal expression, while miR-144 expression was increased. Interestingly, VPA has shown promise in the treatment of some forms of anxiety, such as social anxiety disorder (Kinrys et al. 2003), and has previously also been shown to be useful as an adjunct facilitating the effect of fear extinction, the central mechanism of exposure-based cognitive behavioral therapy (Kuriyama et al. 2011; Whittle et al. 2013). Further investigating functional downstream target genes of miR-34a in primary hippocampal cultures, the authors revealed that administration of a miR-34a inhibitor, as well as lithium or VPA, increased the expression of miR-34a's target gene, metabotropic glutamate receptor 7 (GRM7). Furthermore, administration of an miR-34a precursor led to decreased expression levels of GRM7, suggesting a functional relationship between miR-34a and GRM7 expression levels and pointing toward one novel possibly common neurobiological effect of lithium and VPA with putative therapeutic significance.

Since this breakthrough study, the number of microRNAs linked with relevant downstream effects of therapeutic drugs commonly used in the treatment of anxiety and affective disorders has steadily increased. Antidepressants, such as selective serotonin reuptake inhibitors (SSRI) used as first-line treatments in different anxiety disorders (Baldwin et al. 2014), have been particularly investigated in this regard. Baudry and colleagues were the first to investigate SSRI effects following different modes of administration and revealed increased expression of miR-16 in the raphe nucleus and decreased expression in the locus coeruleus of mice (Baudry et al. 2010). Interestingly, the expression patterns of miR-16 in the raphe nucleus and locus coeruleus were inversely correlated with the expression levels of one of its target genes, the serotonin transporter (SERT). These data indicate that the therapeutic effect of fluoxetine could in part be mediated via differential miR-16 expression levels in monoaminergic neurons targeting SERT in the raphe nucleus and locus coeruleus (Baudry et al. 2010). Using a mouse model of PTSD, oral treatment with fluoxetine for 31 days reduced anxiety-related behavior in these mice, and this was associated with long-term effects on microRNA expression (Schmidt et al. 2013): the expression of miR-1971 was even after 74 days decreased in the prefrontal cortex

in comparison with control groups, suggesting that the long-lasting beneficial effects on anxiety-related behaviors in fluoxetine-treated mice may be mediated in part by reduced miR-1971 expression. Taken together, these studies implicate the regulation of specific microRNA candidates (e.g., miR-16, miR-1971) in distinct brain regions in the therapeutic action of fluoxetine (see also Fig. 1).

Other SSRIs have also been investigated for a possible relation between their behavioral effects and microRNA regulation. It has been shown that maternal separation or chronic unpredictable stress-induced increase in depression-/anxiety-related behavior was associated with decreased levels of miR-326 expression in the nucleus accumbens and striatum (Zhang et al. 2015). Following chronic escitalopram treatment, the levels of miR-326 in both brain areas increased, thus returning to levels comparable with those of non-stressed or saline-treated rats. In investigating effects following tricyclic antidepressant treatment, it was shown that mice treated chronically with imipramine for 18–21 days display lower social avoidance, which was associated with increased expression of miR-135a in the raphe nucleus. As mentioned above, miR-135a targets the serotonergic system directly via binding to the serotonin transporter and 5HT-1a receptor gene (Issler et al. 2014). Demonstrating the functional involvement of miR-135a expression, its overexpression in serotonergic neurons was associated with decreased anxiety- and depression-like behavior following social defeat, while its knockdown led to increased anxiety- and depression-like behaviors (Issler et al. 2014).

Information concerning the effects of antidepressants on microRNA expression in the human brain is scarce. Interestingly, in patient's peripheral blood, the baseline expression of miR-1202 was predictive of treatment success, as patients who responded to treatment exhibited lower blood expression of miR-1202, which subsequently increased following an 8-week treatment with citalopram (Lopez et al. 2014). This brain-enriched microRNA was found to target the GRM4 gene, which shows decreased expression following treatment with antidepressants (Cruceanu et al. 2016). This observation suggests that miR-1202 contributes to antidepressant action via modulation of glutamatergic neurotransmission. Using human postmortem tissues from patients, Lopez and colleagues were able to demonstrate that major depressive disorder is associated with decreased expression of miR-1202 in the prefrontal cortex and in blood (Lopez et al. 2014). To our knowledge, this is the only study that links the dysregulation of a specific microRNA in the brain of psychiatric patients with the expression in blood and that shows dynamic regulation with treatment, thus demonstrating its possible usefulness as a biomarker of treatment success. Importantly, this study demonstrates long-term changes in specific microRNA expression in the brain associated with an affective disorder. In a recent follow-up study, the authors further explored this relationship between peripheral miR-1202 expression levels and brain activity in patients, following an 8-week treatment with desvenlafaxine (Lopez et al. 2017). Using functional magnetic resonance imaging, the authors found that lower peripheral levels of miR-1202 in desvenlafaxine-treated subjects were associated with increased neural connectivity between the posterior cingulate and the parietal, occipital, and prefrontal cortices.

Therapeutic drugs targeting primarily other neurotransmitter and hormone systems besides the serotonergic system have also been examined for the involvement of microRNA candidates in their mode of action. In one study, treatment with the antidepressants agomelatin, which targets melatonin receptors, and tianeptine, which targets glutamatergic, adenosine, and opioid systems, normalized stress-induced changes in microRNA levels in the dentate gyrus, including the expression of miR-181b, miR-9, and miR-411, among others (Patrício et al. 2015). Treatment with the CRHR1 antagonist NBI-27914 was shown to decrease anxiety-like behavior of mice in the elevated plus maze and open-field test. It was found that miR-34b targets the CRHR1 gene and it was thus demonstrated that administration of an agomir for miR-34b into the paraventricular nucleus of the hypothalamus mimicked the behavioral effect of the CRHR1 antagonist and resulted in reduced anxiety-like behavior (Zhu et al. 2017). Also by targeting GABAergic neurotransmission, microRNA expression changes. Administration of the GABA agonist gaboxadol was associated with reduced anxiety-like behavior and increased miR-33 expression in the dorsal hippocampus (Jovasevic et al. 2015). The pharmacological enhancement of therapy-relevant cognitive mechanisms (see also next chapter) via "cognitive enhancers" including the partial NMDA agonist D-cycloserine (DCS) is a more recent avenue for the treatment of anxiety-related disorders (for recent review see Singewald et al. 2015). In exploring a possible involvement of microRNAs in this effect, the expression of 32 microRNAs in the left dorsal hippocampus was found to be regulated after treatment with DCS. This interesting first finding needs to be followed up in more detail also in relation to a possible facilitation of extinction learning (Malan-Muller et al. 2017).

3.8 Non-pharmacological Interventions

Exposure-based cognitive behavioral therapy is a frequently used psychotherapeutic intervention for anxiety disorders. It involves the process of fear extinction, which results in the gradual reduction of fear- and anxiety-related responses to stimuli perceived as threatening to the patient (Abramowitz 2013; Milad and Quirk 2012; Craske and Stein 2016). Meta-analyses of cognitive behavioral therapy used in treating anxiety disorders show that it is efficacious for a number of anxiety-related disorders but has its limitations, including the problem that it produces insufficient long-lasting effects, and thus symptoms can return (Hofmann and Smits 2008; Tolin 2010; James et al. 2015; Gould et al. 2012). Importantly, anxiety patients are often associated with fear extinction deficits (Milad et al. 2009, 2013; Jovanovic et al. 2012; Michael et al. 2007; Duits et al. 2015; Wessa and Flor 2007). Thus, understanding the underlying mechanisms of deficient fear extinction (Graham and Milad 2011; Hill and Martinowich 2016; Baker et al. 2016; Maren and Holmes 2016) and how to overcome this deficiency may hold potential for the development of novel treatment strategies (for review see Holmes and Singewald 2013, Bukalo et al. 2014). Exploring the role of microRNAs in the rescue of deficient fear extinction,

our group recently revealed an increased expression of miR-144-3p in the basolateral amygdala 2 h following fear extinction training. This increase was not seen in the very first phase of extinction training involving mainly fear retrieval, indicating that the effect was specific for extinction learning (Murphy et al. 2017). MiR-144-3p was found to target a number of genes implicated in the activation of plasticity-associated signaling cascades, including MAPK/ERK, PI3K/AKT, and NOTCH, which have been implicated in extinction mechanisms (Herry et al. 2006; Yang and Lu 2005; Dias et al. 2014). Confirming the functional significance of this, viral overexpression of miR-144-3p in the basolateral amygdala was able to rescue impaired fear extinction in extinction-impaired 129S1 mice and to prevent the return of fear in extinction-competent 129S6 mice (Murphy et al. 2017). Regulation of miR-144 in the context of anxiety has also been observed in humans. A recent study in healthy students found that whole blood miR-144 expression increased during the period preceding a stressful event (an effect involving, it is thought, anticipatory anxiety) before returning to baseline levels in the days following (Katsuura et al. 2012). In another study, miR-144-5p expression levels in the blood plasma of patients suffering from depression/anxiety were found to be lower in comparison with healthy controls (Wang et al. 2015). Compellingly, following 8 weeks of psychotherapy, miR-144-5p expression levels increased and were normalized to expression levels seen in healthy controls, suggesting that miR-144-5p expression levels could possibly be useful as a biomarker of treatment success.

While fear extinction is associated with the formation of a new fear-inhibitory memory, it is also important to note that during this process the original fear memory is brought into a labile state where it is subject to updating mechanisms (Flavell et al. 2013). It is during this period that the reconsolidation of the original fear memory can be disrupted or modulated, allowing for the transition to the predominant expression of a newly formed fear-inhibitory memory (Hemstedt et al. 2017). Exploring the role of microRNAs in this process, Lin and colleagues assessed the expression of microRNAs in the medial prefrontal cortex (Lin et al. 2011). Following fear extinction training, the expression of miR-128b was shown to be increased in the infralimbic subdivision of the medial prefrontal cortex (ILPFC). Interestingly, the authors found that miR-128b targets a number of plasticity-associated genes known to be important for fear memory retrieval (Lin et al. 2011). The fear-associated genes targeted by miR-128b include the regulator of calmodulin signaling (RCS), which has been demonstrated to inhibit calmodulin and to negatively regulate protein phosphatase calcineurin activity overall, leading to the disruption of fear memory consolidation/reconsolidation. Thus, increased miR-128b expression during fear extinction was suggested to facilitate the transition from the expression of fear to the expression of this newly formed fear-inhibitory memory. Indeed, overexpression of miR-128b in the ILPFC was able to enhance the effects of fear extinction, thus indicating that increased miR-128b expression could enhance exposure-based cognitive behavioral therapy via enhancing the expression of fear-inhibitory memories.

Beneficial environmental influences have been shown to contribute to successful outcomes of anxiety treatments (Kheirbek et al. 2012). In preclinical models, environmental enrichment, for example, has been used to model positive environmental stimulation, producing anxiolytic effects (Hendriksen et al. 2014; Huttenrauch et al. 2016). Interestingly, Ragu Varman and colleagues demonstrated increased expression of miR-183 and miR-124 in the amygdala following 30 days of environmental enrichment (Ragu Varman et al. 2013). Expression of miR-124 was also regulated in the dentate gyrus in response to environmental enrichment, and this was associated with increased neurogenesis (Brenes et al. 2016). Another non-pharmacological means that can improve anxiety symptomology is physical exercise. Subjecting male mice to voluntary wheel running reduced anxiety-like behavior and suppressed reinstatement of fear measured in their juvenile offspring, demonstrating transgenerational transmission of changes in anxiety-like behavior. The sperm of the mice following voluntary wheel running was found to have decreased expression of miR-19b and miR-455, while miR-133a was increased (Short et al. 2017). Furthermore, long-term exercise in mice, which was associated with increased resilience to stress, led to the decreased expression of miR-124 in the hippocampus and the increased expression of glucocorticoid receptors Nr3c1 and Nr3c1-1f (Pan-Vazquez et al. 2015). Interestingly, a recent genome-wide association study of positive emotion in humans identified a genetic variant proposed to mediate positive emotionality via the nucleus accumbens and miR-181 (Wingo et al. 2017). We have learned from these findings, taken together, that the beneficial effects of established as well as experimental therapeutic interventions for the treatment of anxiety-related disorders are associated with the regulation of microRNAs. As our understanding of microRNA-mediated therapeutic effects increases, novel therapeutic interventions targeting such mechanisms can be developed to improve treatments in anxiety-related disorders.

4 Conclusion and Further Perspectives

Epigenetic mechanisms, in particular microRNA-mediated modulation of gene expression and its dysregulation in disease states, have become a topic of sustained interest in the search for novel understanding and/or development of therapies for some of the most common diseases. In this chapter we have summarized the evidence linking microRNA regulation with anxiety-related behaviors and anxiety disorders. It has been found that specific microRNAs may play various roles in the development, progression, and treatment of anxiety. First studies have provided functional/causal evidence for such a role. Identified microRNAs include miR-15a, miR-17-92, the miR-34 family of microRNAs, miR-101, miR-124, miR-135, and miR-155, which influence anxiety behavior in a brain area-dependent manner. As an interesting example, miR-124 expression was shown to be upregulated in stress-induced anxiety, and its experimental targeted downregulation in the dentate gyrus led to a reduction in anxiety-like behavior, mediated in part via a miR-124a/BNDF

interaction. Such functional knowledge aids the interpretation of associative studies and the identification of false positives. In this context, it was reported that running-induced anxiolysis is associated with hippocampal downregulation of miR-124.

In addition, we have summarized studies that examined how pharmacological and non-pharmacological interventions affect the expression of microRNAs. It was found that SSRIs commonly used to treat many anxiety disorders are associated with altered expression of a number of microRNAs in the brain, such as miR-16 and miR-1971. Non-pharmacological interventions, such as fear extinction, modeling exposure-based CBT, are associated with distinct changes in miR-144 and miR-128b in the BLA and infralimbic cortex of the mPFC, respectively. This new understanding has been aided by recent methodological advancements in assessing microRNAs functionally. In general, the field has moved beyond correlative studies whereby the presence of disease is associated with the altered expression of microRNA(s) and toward a more functional/causal understanding of microRNAs in distinct brain regions and of their roles in anxiety. Hence, the current data suggest that microRNAs may (in part) mediate therapeutic effects of established drugs and psychotherapeutic approaches used currently to treat these disorders.

This relation suggests that microRNAs could possibly be specifically targeted in the treatment of anxiety, although the therapeutic use of microRNAs for psychiatric diseases is in its very early stages. The initial problem encountered in such an approach – the fact that microRNAs have multiple targets – may in fact be an advantage, given that deregulation in anxiety disorders involves networks rather than single effector molecules. Thus, by targeting a single "key" microRNA, it is possible to modulate entire relevant gene networks. Target specificity may be determined by the observed highly distinct regional, cellular, and temporal expression of some of the candidate microRNAs in relevant nodes of the affected network. Along these lines, microRNA-based interventions targeting candidates such as miR-128b and miR-144 have the potential to augment the efficacy of exposure-based CBT through targeting plasticity-associated genes in extinction learning relevant brain areas such as the infralimbic cortex and basolateral amygdala, respectively. However, there is much work to be done before any microRNA-based therapy for anxiety can be used in a clinical setting. Answers to questions regarding oral bioavailability, the ability of microRNA-based therapeutics to cross the blood-brain barrier, and the presence of off-target effects currently elude us and are required to be solved before a push for microRNA-based interventions can be attempted. Therapeutic methodologies targeting microRNAs, such as virally mediated gene therapy or synthetic locked nucleic acid (LNA) agomirs/antagomirs, are associated with increased risks and thus require careful safety studies that consider off-target effects and other potential problems (Scott et al. 2015). In an effort to circumvent the limitations of virally mediated interventions, targeting mRNAs via locked nucleic acid probes, which are chemically modified to increase thermal stability and hybridization affinity, is a novel and possibly less invasive method of microRNA administration.

It is important to note that while to date numerous microRNAs have been implicated in anxiety, there is often little overlap within animal studies or between animal and human studies. This is in part due to the various experimental setups,

the different behaviors assessed, and the different time points investigated. An important step forward would be to elucidate emerging microRNA candidates that play a role across various experimental setups and behaviors. Furthermore, it is unlikely that a single microRNA candidate alone will be critical in the development, progression, or treatment of anxiety; rather, a specific microRNA "signature" is more likely to be of importance in the various stages (Scott et al. 2015). In support of this, studies have revealed microRNA signatures for cancer, and more recently such signatures have emerged in preclinical models of stress-related disorders (Balakathiresan et al. 2014; Jiang et al. 2015).

In summary, we have provided an update on the most recent research functionally linking microRNAs with anxiety-related behaviors. In particular, it is through these more functional studies that we will be able to move forward in search of novel therapeutic interventions for psychiatric disorders such as anxiety disorders. A more complete regional, temporal, and subcellular picture of the role of microRNAs in anxiety will have the potential to open up novel ways of using this knowledge in the development of microRNA-based therapeutics for the improved treatment of patients suffering from anxiety-related disorders (Aten et al. 2019; Hommers et al. 2018; Sun et al. 2019; Snijders et al. 2018).

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Transposable Elements

G. Guffanti, A. Bartlett, P. DeCrescenzo, F. Macciardi, and R. Hunter

Contents

Abstract Transposable elements (TEs) are low-complexity elements (e.g., LINEs, SINEs, SVAs, and HERVs) that make up to two-thirds of the human genome. There is mounting evidence that TEs play an essential role in molecular functions that influence genomic plasticity and gene expression regulation. With the advent of next-generation sequencing approaches, our understanding of the relationship between TEs and psychiatric disorders will greatly improve. In this chapter, the Authors comprehensively summarize the state-of the-art of TE research in animal models and humans supporting a framework in which TEs play a functional role in mechanisms affecting a variety of behaviors, including neurodevelopmental, neuropsychiatric, and neurodegenerative disorders. Finally, the Authors discuss recent

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therapeutic applications raised from the increasing experimental evidence on TE functional mechanisms.

Keywords Epigenomic regulation · Exapation · Neuropsychiatric disorders · Therapeutic targeting · Transposable elements

1 Introduction

Transposable elements (TEs) refer to a class of DNA sequences that can move to different locations within the genome. Current estimates are that TEs comprehensively make up to between half (Kuhn et al. 2007; Lander et al. 2001) and two-thirds (de Koning et al. 2011) of the human genome. These elements consist of low-complexity sequences that are classified into two major classes based on their mechanism to copy or move throughout the genome: retrotransposons, also called class I, and DNA transposons, class II. Class I TEs function through a copy-paste mechanism requiring an RNA intermediate, endonuclease, and reverse transcriptase for transposition (Wang and Kunze 2015). In contrast, class II TEs transpose via a cut-and-paste mechanism requiring only endonuclease activity for excision and insertion (Wang and Kunze 2015). Class I TEs are further classified into two groups based on the presence or absence of long terminal repeats (LTRs). The majority of TEs are represented by non-LTR retrotransposons that include long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), and SINE-VNTR-Alu (SVAs). In particular, there are three major classes of LINEs (up to 6 K bp), LINE-1, LINE-2, and LINE-3, of which only LINE-1 retained the ability to transpose in the contemporary genome (Brouha et al. 2003). SINEs are the second most abundant class of TEs after LINEs, are relatively shorter (300 bp), and are mostly represented by the Alus family. SVA elements are structurally complex, derived from different genomic repeats, an Alu-like sequence, and a SINE with a size ranging between 600 and 4 K bp. LTR transposons are represented by endogenous retroviruses (ERVs), characterized by long sequences (up to 10 K bp), although the majority of elements are truncated. Believed to be remnants of ancient germline viral infections, ERVs are especially active prenatally and may play a critical role in neurodevelopment. Finally, DNA transposons are apparently not mobile. Their sequence includes a protein called transposase, which is bound by terminal inverted repeats (TIRs) responsible for the excision and reintegration of the elements into the genome.

With the advent of modern molecular tools, such as RNA sequencing, to detect and classify TEs on genome-wide scale, it became possible to further study their distribution and functional role. It is now apparent that TEs may play a critical role in molecular processes that influence genomic plasticity and gene expression regulation. Although further evidence is needed to more fully establish the nature and mechanisms of their functions, emerging evidence supports TEs as a novel and still largely understudied category of noncoding RNAs with putative regulatory role. In

this chapter, we will review the main principles of TE origins and biogenesis and then present our current understanding of their mechanisms of function. Then, we review evidence from preclinical and clinical studies supporting a role for TEs in neuropsychiatric, neurodevelopmental, and neurodegenerative disorders. Finally, we review what is known about TEs as drug targets and their potential relevance for pharmacological treatments.

2 Origins and Biogenesis

2.1 Evolution

TEs are present in most known organisms, including prokaryotes. However, in prokaryotic organisms, they comprise a relatively small fraction of a genome, mainly containing protein-coding genes. In many multicellular eukaryotes, the opposite is true, and TEs often represent the most substantial fraction of the genome by order of magnitude (Fedoroff 2012; Goodier and Kazazian 2008). TEs are now known to be a significant influence on genome structure and evolution in most eukaryotic species (Fedoroff 2012). While TEs are present in most taxa, it is only in eukaryotic organisms that they have come to comprise a majority of the genome. In prokaryotes, the selective pressures imposed by the need for high rates of replication ensure that genomes remain small. Further, prokaryotes lack the bioenergetic capacity to support large genomes. Lane and Martin have argued that the mitochondrial endosymbiosis at the root of eukaryotic evolution permits higher ratios of energy production per gene and thus more extensive, complex genomes and cells (Lane and Martin 2010). Protein production represents the most considerable part of cellular energy budgets (Harold 1986). Mitochondrial endosymbiosis permitted the expression of roughly five orders of magnitude more protein than is possible for prokaryotes. This energetic wealth also meant that eukaryotic genomes could support many more competitive noncoding elements than was possible in prokaryotes. This would, of course, have placed pressure on eukaryotes to develop mechanisms to control these potentially parasitic elements, and that control, in turn, may have permitted the evolution of extensive regulatory systems based on noncoding RNAs (ncRNAs) derived from TEs as well as other features of complex eukaryotic genomes such as molecular epigenetic modifications of histones (Bartlett and Hunter 2018; Daskalakis et al. 2018; Fedoroff 2012; Friedli and Trono 2015; Hunter et al. 2013, 2015; Wheeler 2013).

The mitochondrial endosymbiotic event is thought to have driven the entry of type II introns, arguably the earliest group of transposon-like elements, into eukaryotic genomes. These were thought to derive from the mitochondrial genome itself and likely accompanied the transfer of most of the mitochondrial coding genome to the nuclear genome. It is likely that many other TEs also entered eukaryotic genomes during this early phase in eukaryotic genome evolution, perhaps including the LINEs, whose origin is ancient but as yet poorly understood. Given the recent

finding that LINE-1 is required for the transition from the two-cell stage to the blastocyst during embryonic development in mammals (Percharde et al. 2018), it seems likely that the LINEs may have entered eukaryotic genomes around the time that multicellularity emerged about 600 million years ago (Eickbush and Jamburuthugoda 2008). Other elements appear to have derived from the host genome itself. Most SINEs derive from ribosomal or transfer RNAs. The primate Alu SINEs, for example, derive from 7SL RNAs (Ullu and Tschudi 1984). Long terminal repeat TEs and endogenous retroviruses derive from ancient retroviral infections, some relatively recent and undomesticated. Murine leukemia virus (MLV) retroelements entered the murine genome roughly 1.5 million years ago (Boeke and Stoye 1997), and intracisternal A-particle (IAP) elements, which also entered murid genomes in the past few million years, still have members capable of generating virus particles (Ribet et al. 2008).

In contrast to protein coding genes, TEs are often not conserved across even closely related taxa. Species within the same genus may have different and unique classes of TEs in their genomes. Chimpanzees, for instance, have two families of TEs not present in the human genome (Polavarapu et al. 2006). TEs are likely to be significant drivers of speciation events due to their capacity to drive both large-scale changes in chromosomal organization and to their capacity to create new genes and new chromatin landscapes. Evidence suggests that bursts of transposon activity accompany many speciation events (Belyayev 2014; Fedoroff 2012; Lane and Martin 2010; Senerchia et al. 2015). These differences mean that both the total percentage of the genome occupied by TEs and the clades of TEs differ substantially across species, contributing to pronounced differences in genome size and function (Fedoroff 2012). Similarly rates of TE insertion and deletion vary considerably between species. Drosophila shows fairly high rates of TE insertion (as high as one per meiosis) for I elements (Bucheton et al. 1992), yet the fraction of the Drosophila genome occupied by TEs is roughly 4% (Fedoroff 2012) due to the effects of selection and deletion (Eickbush and Furano 2002). In contrast, humans and mice have much larger fractions of their genomes occupied by retrotransposons (Lander et al. 2001; Mouse Genome Sequencing Consortium et al. 2002) and a similar rate of insertion to the fruit fly, though there is a much lower rate of deletion (Eickbush and Furano 2002).

2.2 Exaptation

While it has long been argued that transposons are either genomic parasites or junk, the evidence is accumulating that they have acquired novel functional roles in a number of instances in many if not most multicellular genomes. This type of phenomenon is usually referred to as “exaptation,” a terminology initially introduced to describe the acquisition of a new function of a trait during evolution and then used for DNA sequences such as TEs (Gould and Vrba 1982). The most persuasive

argument that most of the genome is junk was articulated by Susumu Ohno's calculation that the genome could contain on the order of 20,000 selectable loci, based on observed rates of deleterious mutations (Ohno 1972). However, the finding that more than 80% of the genome is transcribed raised doubts as to the extent that Ohno's hypothesis was correct, at least beyond those parts of the genome that code for protein (ENCODE Project Consortium 2012). The underlying assumptions of Ohno's argument are founded in the biochemical constraints imposed by the genetic code, which requires a high level of sequence fidelity in order to avoid producing a codon which codes for an inappropriate amino acid and thus causes a defect in the structure of the translated protein. RNA genes, which do not have this constraint, would not necessarily suffer to the same degree from a single point mutation. Indeed, it appears that ncRNA genes are under selection pressure for structure, rather than sequence (Nitsche and Stadler 2017), and some estimates have put the number of selectable ncRNA genes as high as four million (Smith et al. 2013). As roughly 80% of confirmed ncRNA sequence is TE-derived, it follows that much if not most expressed TEs may be functional (Kelley and Rinn 2012). While this likely represents a high bound on the number of functional ncRNA elements in the genome, it nonetheless represents an essential argument for the function of TEs specifically and ncRNA in general (Bartlett and Hunter 2018; Daskalakis et al. 2018).

We have argued elsewhere that the transposome represents an "adaptive toolkit" for metazoan genomes, perhaps providing a replacement for the extensive lateral gene transfer present in prokaryotic organisms (Bartlett and Hunter 2018; Hunter et al. 2015; Lapp and Hunter 2016; Sonea and Mathieu 2000). Though at present the extent to which the transposome is functional is a major undecided question in biology, it is clear that TEs have been exapted to useful purpose in a number of cases. The V(D)J recombination system responsible for the generation of antibody diversity in the mammalian immune system is the result of the domestication of retrotransposons to useful purposes (Carmona and Schatz 2017), and the placenta also derives from exapted transposons (Dupressoir et al. 2012; Frank and Feschotte 2017; Lynch et al. 2011, 2015). Within the nervous system, LINE-1 retrotransposons have been shown to contribute to neuronal diversity during development and adult neurogenesis (Erwin et al. 2014) and have been shown to be regulated in part by environmental stress and maternal care (Bedrosian et al. 2018; Muotri et al. 2009). The mouse B2 SINE, which acts as a transcriptional regulator through a direct interaction with RNA polymerase II, is regulated in the brain by both stress and heat shock (Hunter et al. 2012; Kugel and Goodrich 2006; Ponicsan et al. 2010). Perhaps the most persuasive argument for the functional importance of retrotransposon RNA comes not from the brain but from studies of early development. LINE-1 appears to be required for successful transition from the 2–4 cell stages of embryonic development (Percharde et al. 2018), noted above. Whether or not this requirement for TE expression is not established for other developmental stages, it seems likely that the transposome will prove to play a role in more, rather than less, of our biology, the more it is explored.

3 Mechanisms of Function

Barbara McClintock first described DNA transposons as "controlling elements" regulating the "time and type of activity of individual genes" (Mcclintock 1956). While initially disregarded as "junk," the regulatory properties of TEs are recently of increased focus (Chuong et al. 2017). Since their initial observation, much evidence has accumulated suggesting exaptation of TEs both at the level of genomic structure and transcriptional regulation. Recent studies describe the role of TE in influencing genomic plasticity as well as their exaptation into transcriptional regulators such as new promoters, enhancers or insulators, and sources of new transcription factor binding sites (TFBS), respectively.

Genomic Plasticity Despite the genome's set up mechanisms to regulate TE activity, some TEs escape repression and introduce new insertions both in germline cells during embryonic development and in somatic tissues later in life. While the majority of LINE-1 referenced in the genome annotation is retrotranspositionally inactive, there are about 100 retrotransposition-competent LINEs in an average diploid human genome (Brouha et al. 2003). The rate of LINE-1 retrotransposition in humans is between 1/270 and 1/95 births (between 0.3 and 1%, respectively) (Ewing and Kazazian 2011). New TE insertions can affect genome integrity in several different ways (Cordaux and Batzer 2009; Guffanti et al. 2014). First, TE insertions in exonic regions can often lead to gene dysregulation and damage (Wang and Kunze 2015). Second, TEs insertion can create new exons or new genes by carrying along non-retrotransposon DNA sequences (Sela et al. 2010). Third, TEs can lead to a phenomenon called transduction, which refers to the duplication of the 3′ or 5′ flanking sequence along with the new copy of the original TE (Goodier et al. 2000; Pickeral et al. 2000; Tica et al. 2016). Fourth, TEs can induce LINE-1 nonallelic homologous and nonhomologous recombination events resulting in significant duplication and deletions of genomic DNA sequences (Campbell et al. 2014; Startek et al. 2015). Finally, TEs have been implicated in the generation of microsatellites from homopolymeric genomics tracts (Ahmed and Liang 2012). Overall, the role of TE in influencing genomic plasticity has been recognized as one of the major driving forces of the emergence of copy number variations (CNV) (Bose et al. 2014), whose effects have been widely associated to diseases of the nervous system (Kirov 2015).

Transcriptional Regulation While not yet fully elucidated, an emerging role for TEs in regulation of biological processes is increasingly evident. Beyond the genomic effects of these elements, a role for the RNA of class I elements is emerging (de Souza et al. 2013; Long et al. 2017). Several possible mechanisms for retrotransposon RNA in transcriptional regulation have been hypothesized. These include RNA recruitment of protein complexes and RNA inhibition by acting as a "decoy" (i.e., transcription factor "sink"), indirectly through the act of transcription itself and through nucleosome organization. Central to this type of regulatory mechanisms is the binding of transcription factor (TF) proteins to specific functional

sequences, also referred to as "motifs," within TEs. The presence of functional motifs within TEs has been documented for over 20 years (Brosius 2003). In each case, class I TEs may play a critical role in rewiring the complex dynamic of transcriptional regulation.

Promoters Derived from TEs Most TEs contain *cis*-regulatory sequences that function as promoters. In addition to providing the elements recognized by host cell machinery to control TE transposition (Chuong et al. 2017), these sequences have the potential to be exapted as promoters for neighboring genes. For example, LTRs, which are HERVs promoter sequences flanking the coding elements essential for viral replication, have the ability to recruit RNA polymerase II (Pol II), which is the enzyme essential to initiate transcription (Mager and Stoye 2015). Fully autonomous LINEs capable of retrotransposition feature Pol II promoter sequences in the 5′ UTR as well as an antisense promoter. Similarly, SINEs which are thought to have evolved from Pol III-transcribed genes contain Pol III-specific recruitment sequences (Batzer and Deininger 2002). For example, active human Alu SINE elements contain both a Pol III promoter and Pol III-specific stop sequence (Batzer and Deininger 2002; Chu et al. 1995). These sequences may drive the expression on neighboring genes once exapted into alternative promoters (Richardson et al. 2015; Speek 2001; Swergold 1990). SVAs represent an evolutionarily young, primate-specific, composite retrotransposon featuring elements of Alu SINE, R-SINE, and VNTRs (Lapp and Hunter 2016; Shen et al. 1994). Beyond structural homology, hallmarks of SVAs include a polyadenylation signal at the 3′ end as well as recognition sequences for LINE-1 endonuclease suggesting that LINE-1 activity likely facilitates SVA abundance (Hancks et al. 2011). Interestingly, these elements appear relatively intact compared to LINE-1 elements which largely feature 5′ truncation (Wang et al. 2005).

TEs appear to have coevolved with host systems to respond to environmental stimuli including heat shock, infection, and stress (Hunter et al. 2012; Karijolich et al. 2015; Steller and Pirrotta 1986). Interestingly, single-cell RNA-sequencing has shown that B2 SINE expression increases following neuronal activation in vitro (Lacar et al. 2016). The heat shock response induces large changes in the transcription upregulating heat shock proteins while downregulating expression of housekeeping genes (DiDomenico et al. 1982; Findly and Pederson 1981; Gilmour and Lis 1985; O'Brien and Lis 1993). B2 SINE expression is increased as much as 40-fold as part of this response (Fornace and Mitchell 1986; Li et al. 1999; Liu et al. 1995). When B2 expression is inhibited, the heat shock response becomes dysregulated (i.e., housekeeping genes remain upregulated) (Allen et al. 2004). B2 SINE accumulation appears to be permissive for selectively downregulating Pol II-driven expression (Espinoza et al. 2004). This group also found that B2 RNA directly interacts with Pol II through the 3′ stem loop and inhibited transcription via single-stranded regions (Espinoza et al. 2007). Interestingly, Alus, which are similarly upregulated in response to heat shock, also appear to bind Pol II and inhibit transcription (Liu et al. 1995; Yakovchuk et al. 2009).

Despite the large fraction of the genome occupied, many of the TEs scattered throughout the genome are truncated and incapable of replication; yet the implications of the regulatory elements they still possess remain not fully realized (Feschotte et al. 2002; Lander et al. 2001).

Enhancers Derived from TEs TEs can be exapted into enhancers by donating sequences that can regulate the expression of neighbor genes. Enhancers are constituted by an array of transcription factor binding sites (TFBS) and are usually located far away from the promoter region of their target gene. Also defined as distal regulatory regions, enhancers regulate the expression of genes by interacting with transcription factors. The first evidence of TE exaptation into enhancers started to appear in the early 1990s and implicated mostly SINE (Hambor et al. 1993; Santangelo et al. 2007) and LINE (Yang et al. 1998). More recently, genome-wide investigation allowed to better characterize the TE-derived landscape of enhancers. Marino-Ramirez and colleagues found that TE sequences reside in 23% of regions of DNaseI-hypersensitive sites (DHS), which indicate regions of open chromatin involved in the regulation of transcription typical of promoters and enhancers. TE sequences in these regions displayed a high degree of correlation with nearby genes in CD^{4+} T cells (Mariño-Ramírez and Jordan 2006). Furthermore, genome-wide histone modification analysis in two cell types from the ENCODE project predicted the locations of thousands of TE-derived enhancers, with a minimum (~3%) overlap between the two cell types (Huda et al. 2011). These findings indicate that TE-derived sequences have the potential to be exapted into enhancers in a cell-type specific way and that most likely the enhancer-like function is mediated by epigenetic regulatory mechanisms. Another study by the same group of researchers showed that MIRs, one of the most ancient classes of TEs, were evolutionary co-opted into enhancers with a wide range of TFBS sequence motifs, including those associated with transcription factors C-JUN, NF-E2, and ZNF274 (Jjingo et al. 2014). A few, but consistent findings, revealed that HERVs might act as enhancers of protein-coding gene expression, for example, ERVs of the family -H have been shown to act as scaffold to recruit p300 and OCT4 to drive the expression of neighboring genes in the maintenance of stem cell identity (Lu et al. 2014); the ERV sequence at the promoter region of *PRODH* gene, previously associated with schizophrenia, has been shown to upregulate its expression when found in an under-methylated state (Suntsova et al. 2013).

Insulators Derived from TEs TEs can become insulators of a nearby transcriptional unit either by preventing the spread of heterochromatin from one locus to another or by acting as enhancer blockers. Perhaps the most persuasive example of this mechanism is provided by B2 SINE acting as transcriptional as well as epigenetic roadblocks at the murine growth hormone (GH) locus, silencing its expression (Hambor et al. 1993; Santangelo et al. 2007). The mouse growth hormone (GH) gene does not contain a canonical locus control region but is flanked by a B2 SINE element. Deletion of the B2 SINE element upstream of the GH transcription start site suggested that the B2 SINE was necessary for developmentally regulated GH expression. During mouse embryogenesis, levels of H3K9me3 proximal to GH

locus in the pituitary decline while H3K9me2 levels increase. Specifically, H3K9 valence transitions at the B2 SINE element upstream of the GH gene. This B2 SINE features flanking Pol II and Pol III promoters that appear permissive for overlapping transcription of the element. Pol III transcription appears to facilitate euchromatic H3K14 acetylation, while Pol II transcription prevents H3K9me3 maintenance (Lunyak et al. 2007). This demonstrates a dynamic role for the B2 SINE element in both acting as a Pol II transcriptional roadblock and regulating local chromatin structure.

New Regulatory Regions Derived from TEs Retrotransposon RNA inhibition of transcription factor binding has not yet been determined. However, a number of emerging lncRNAs have been shown to act as "decoys" for protein regulators of transcription (Long et al. 2017). For example, the lncRNA Gas5 acts as a "riborepressor" through interaction of its glucocorticoid response element (GRE) with the DNA-binding domain of the glucocorticoid receptor (Kino et al. 2010). The GRE contained within the Gas5 RNA serves to sequester available receptors from genomic interaction. Given the abundance of transcription factor motifs found in class I TEs, it stands to reason that transcription factors bind similarly to retrotransposon RNA. As methods for preserving RNA-protein immunoprecipitation continue to improve, it is likely that "decoy" TE RNAs will be identified (Mili and Steitz 2004).

4 Preclinical Studies

Evidence for associations between TE activity and human brain disorders is fairly extensive (Guffanti et al. 2014; Reilly et al. 2013), but data from preclinical models of these diseases remains relatively sparse.

Stress contributes to many human neuropsychiatric diseases, and several studies have demonstrated stress-induced regulation of TEs in the brain (Hunter et al. 2013). Evidence is emerging that stress, maternal behavior, and exercise alter rates of transposition in the mouse hippocampus (Bedrosian et al. 2018; Muotri et al. 2009). Exposure to acute stress drives downregulation of IAP-LTR and B2 SINE in the rat hippocampus and upregulation of IAP-LTR and LINE-1 in the cerebellum. Further, in the hippocampus, global increases in the repressive histone mark H3K9me3 was observed across a large number of retrotransposon loci (Hunter et al. 2012). Recently, it was observed that stress increases the N6 methyladenine modification in mouse prefrontal cortex, roughly half of which seemed to be targeted to LINEs and associated with a decline in RNA expression from those elements (Yao et al. 2017). Cappucci and colleagues also observed strain-specific changes in LINE-1 expression in the mouse hippocampus after 5 days of repeated stress (Cappucci et al. 2018). Another study found that at least a subset of LINEs are also transcribed under normal conditions in the rat hippocampus, suggesting a role in nonstress neuronal physiology as well (Mukherjee et al. 2016). As noted in the

section on exaptation, heat shock upregulates B2 SINE RNA expression in the mouse brain (Kugel and Goodrich 2006).

Cortical spreading depression (CSD) is associated with disorders such as migraine and epilepsy. In rodent models, CSD has been associated with changes in both DNA methylation and H3K9me3 of LINE1 in cortical genomes (Drongitis et al. 2016; Rana et al. 2012).

The possibility that retrotransposons play a role in psychotic disorders dates to at least the 1980s (Crow 1987), and the idea has received increasing empirical support with the advent of modern genomic approaches. While animal models of psychotic disorders have substantial limitations (Kesby et al. 2018; Mattei et al. 2015), there is nonetheless preclinical evidence supporting a role for retroelements in the pathogenesis of psychosis. One example is the finding that the insertion of a MusD retroelement into the *Slc6a5* gene induces a behavioral phenotype similar to that of other rodent schizophrenia models (Bogdanik et al. 2012). Overexpression of HERV-W env in U251 glioma cells alters expression of two genes associated with risk of schizophrenia, brain neurotrophic factor (*BNDF*) and the dopamine D3 receptor (*DRD3*) (Huang et al. 2011). Exogenous infections with cytomegalovirus and *T. gondii* can activate HERV-W transcription in cultured fibroblasts and neurons, respectively (Frank et al. 2006; Nelson et al. 1999). Interestingly, valproic acid treatment increases the expression of HERV elements in human cell lines, perhaps via its effects on histone acetylation (Diem et al. 2012).

TEs have also been proposed to play a role in neurodegenerative disorders. In Drosophila models of ALS and frontotemporal dementia, transposon activation via TDP-43 mediated suppression of small-RNA-mediated silencing (Krug et al. 2017; Li et al. 2012, 2013). These findings have found further support from work in both human cells and chicks (Li et al. 2015; Voigt et al. 2010). Deletion of the Uhrf1 gene in the developing mouse cerebellum results in severe neurodegeneration due to overexpression of the murid IAP endogenous retrovirus (Ramesh et al. 2016). Interestingly, mercury, which can induce neurodegeneration, causes increases LINE-1 expression in human neuroblastoma lines (Habibi et al. 2014). Perhaps most significantly, dysregulation of Alu expression has been demonstrated to mechanistically contribute to macular degeneration in both humans and model systems (Kerur et al. 2013; Tarallo et al. 2012). The latter studies demonstrate that disruption of small-RNA-mediated silencing of SINE RNA via downregulation of Dicer-1 expression results in degeneration via an NLRP3 inflammasome-mediated pathway.

These findings support the idea that TEs have a role to play in a variety of brain disorders. The observational data in patient populations is increasing in depth and strength as well. However, much further research is needed to build strong preclinical models of TE-driven pathology if we are to develop a clear mechanistic understanding of diseases induced by TEs.

5 TEs in Development and Disease

There is an emerging link between TEs and psychiatric disorders. TEs have been implicated in (1) neuropsychiatric disorders, such as schizophrenia (Weis et al. 2007), bipolar disorder (Weis et al. 2007; Yolken et al. 2000), major depressive disorder (MDD) (Weis et al. 2007), post-traumatic disorder (PTSD) (Rusiecki et al. 2012), and substance use disorders (Okudaira et al. 2014; Ponomarev et al. 2012); (2) neurodevelopmental disorders, such as attention deficit hyperactivity disorder (ADHD) (Balestrieri et al. 2014; D'Agati et al. 2016), autism (Balestrieri et al. 2012), and Rett syndrome (Muotri et al. 2010); and (3) neurodegenerative disorders, such as Alzheimer's disease (Huang et al. 2006), frontotemporal lobar degeneration (FTLD) (Li et al. 2012), ataxia telangiectasia (Coufal et al. 2011), and X-linked dystonia parkinsonism (Hancks et al. 2011; Makino et al. 2007). Here, we discuss the most significant findings.

5.1 Neuropsychiatric Disorders

Schizophrenia Multiple studies have shown differences in the expression of TEs both in neural tissue, blood, and cerebrospinal fluid (CSF) between people with schizophrenia and healthy controls. Yolken et al. (2000) first discovered a link between ERVs and schizophrenia (Yolken et al. 2000). Specifically, they found an increase in MSRV (multiple sclerosis retrovirus), homologs of ERVs initially identified in the body fluids of individuals with multiple sclerosis and in the postmortem frontal cortical tissue taken from people with schizophrenia, relative to healthy controls. This finding was followed by the detection of increased levels of human ERV-W by the same group of researchers, both in postmortem brain tissues and CSF (Karlsson et al. 2001). In an additional follow-up study, the researchers found a statistically significant increase in levels of the *gag* protein of ERV-W in the plasma of people with schizophrenia-spectrum disorders (schizophrenia, schizoaffective psychosis, and schizophreniform disorder) compared to healthy controls (Karlsson et al. 2004). Several other studies reported on the increased transcriptional activation of different types of ERVs in schizophrenia patients, like HERV-K10 in neural tissue (Frank et al. 2005), HERV-W in blood (Huang et al. 2006, 2010; Perron et al. 2012), and in postmortem anterior cingulate gyrus (ACG), and in many hippocampal structures (Weis et al. 2007). In a follow-up study, Huang et al. (2011) transfected human glioma cells with pCMV-*env*, a plasmid containing an HERV-*env* sequence. They found that increases in the copy number of HERV-W transcripts were associated with increased expression of BDNF, DRD3, NTRK2, and CREB in vitro (Huang et al. 2011). This finding represents the first evidence suggesting that retroviral RNA can impact the expression of genes involved with the development of schizophrenia. All of these studies were conducted focusing on one

specific family/type of TEs, based on a study design characteristic of candidates selected a priori by the researchers.

With the advent of new technologies, and specifically next-generation whole genome-sequencing, it was finally possible to extend the investigation to the entire genome of schizophrenia patients in different tissues and in some cases even in single neurons. Bundo et al. (2014) found an increase in LINE-1 insertions in neurons from postmortem prefrontal cortex of schizophrenia patients and in induced pluripotent stem cell (iPSC)-derived neurons containing 22q11 deletions, a known genetic risk factor for the disease (Bundo et al. 2014). Many of these insertions were mapping to genes related to synapse formation and to genes whose mutations are associated with schizophrenia. Further, using animal models, they demonstrated that environmental triggers, such as perinatal environmental risk factors, can lead to increase LINE-1 retrotransposition. This finding corroborates the hypothesis that a prenatal genetic vulnerability might be exacerbated by an environmental risk exposure later in life, also known as the two-hit model of schizophrenia. LINE-1 insertions in schizophrenia-relevant genes were also detected in the blood DNA in a schizophrenia-affected family (Guffanti et al. 2016). This was the first evidence of Mendelian inheritance of LINE-1 insertions, very similar to any other structural genomic variant. Most of these insertions were human-specific LINE-1 (L1HS) insertions, mapping ubiquitously within and outside known genes and ncRNAs. Doyle et al. (2017) found an increase in intragenic LINE-1 insertions in the DNA derived from postmortem dorsolateral prefrontal cortical tissue taken from individuals with schizophrenia compared to healthy controls (Doyle et al. 2017). These LINE-1 insertions were primarily found within genes related to the postsynaptic membrane and to cell projections, suggesting that LINE-1 insertions in these genes may lead to a disruption of the development or function of these neuronal structures (Doyle et al. 2017).

It is worth noting that most of the patients with schizophrenia-spectrum disorders who take part in these studies have been on psychotropic medication at some point in their lives and that psychotropic medications have been shown to be among the potential triggers of TE expression. In conclusion, the evidence reviewed so far support the hypothesis that TEs such as LINE-1s and human ERV-Ws contribute to the development of schizophrenia. It is also possible that an exogenous infection, such as a virus or infection, serves as a trigger for both inflammation and endogenous retroviral activity in the pathogenesis of schizophrenia (Karlsson et al. 2004; Leboyer et al. 2013).

Bipolar Disorder The link between TEs and bipolar disorder (BP) was first noted by Yolken et al. (2000), who found an increase in the expression of human ERV-K in postmortem frontal cortical tissue taken from patients with bipolar disorder relative to healthy controls (Yolken et al. 2000). Frank et al. (2005) also found an increase in HERV-K10 expression in neural tissue from patients with BP (Frank et al. 2005). Weis et al. (2007) found that people with BP displayed increased expression of the *gag* protein of human ERV-W compared to controls in many hippocampal structures (Weis et al. 2007). Perron et al. (2012) found increased

expression of the *env* protein of human ERV-W in people with BP relative to controls, coupled with a lower genomic copy number of the *env* protein (Perron et al. 2012). Interestingly, subjects with BP had increased *env* expression both in relation to healthy controls and to patients with schizophrenia. Given that environmental input can trigger *env* expression, the combination of its increased expression and reduced copy number in patients with BP could be related to an infectious or viral trigger, which could lead to increased activation of this TE.

MDD and PTSD Weis et al. (2007) found decreased expression of human ERV-W (*gag* protein) in the postmortem anterior cingulate gyrus, as well as in the CA2 interneurons, axons, and dendrites of the hippocampus of major depressive disorder (MDD) patients compared to healthy controls (Weis et al. 2007). Rusiecki et al. (2012) assessed the overall methylation of LINE-1s and Alus in US soldiers before and after deployment (Rusiecki et al. 2012). Cases were defined as soldiers who developed PTSD after deployment; controls were defined as soldiers who did not develop PTSD after deployment. Soldiers with any other psychiatric disorder were excluded from this study. There was no difference in serum LINE-1 methylation between cases and controls before deployment. After deployment, LINE-1 methylation in controls increased, while it stayed at baseline levels in cases. The authors speculate that the increase in LINE-1 methylation, and probable consequent decrease in LINE-1 expression, under extreme stress serves as a protective factor for the development of PTSD. Before deployment, cases had greater Alu methylation than controls. After deployment, cases had increased methylation relative to their pre-deployment levels and relative to control post-deployment levels. Although these results weren't statistically significant, they are worth noting as this represents a first-of-its-kind finding. This study indicates the possibility that serum Alu hypermethylation and LINE-1 hypomethylation might increase vulnerability to the effects of psychological stress, while Alu hypomethylation and LINE-1 hypermethylation might increase resilience in the face of it.

Substance Use Disorders Ponomarev et al. (2012) analyzed TEs in postmortem amygdala and superior frontal cortex taken from individuals with alcohol use disorder (AUD) and healthy controls (Ponomarev et al. 2012). They found an increase in the expression of long terminal repeats (LTRs), which represent the promoter sequences of human ERVs, in patients with AUD, coupled with a decrease in methylation. Overall, this evidence suggests that the increase in LTR expression might be related to reduced gene silencing. While it is possible that human ERV hypomethylation is a predisposing factor for the development of AUD, previous research suggests that such hypomethylation could also be a consequence of alcohol use. The authors of the study also noted that human ERV overexpression might increase the levels of the human ERV-encoded genes and the envelope glycoprotein gene (*env*), which have been associated with neurotoxic effects of alcohol, including cytotoxicity to oligodendrocytes and myelin degeneration.

Another study links TEs to neurotoxicity, in which LINE-1 retrotransposition is induced by several neurotoxic drugs. Okudaira et al. (2014) found that treating human neuroblast cell lines with methamphetamine caused LINE-1 retrotransposition

(Okudaira et al. 2014). There was no LINE-1 retrotransposition in non-neuronal cells in response to methamphetamine. Cocaine also induced LINE-1 retrotransposition in neuronal cell lines. In another experiment, D4T, a compound that inhibits reverse transcriptase, prevented LINE-1 retrotransposition when neuronal cell lines were exposed to methamphetamine and to cocaine. Additionally, the authors demonstrated that neither methamphetamine nor cocaine induced LINE-1 retrotransposition when CREB functional activity was silenced via specific siRNA (Okudaira et al. 2014). This finding suggests that CREB phosphorylation is also critical for LINE-1 retrotransposition in response to these drugs. Further experiments indicated that the open reading frame 1 (ORF1) of LINE-1s is recruited and translocated to chromatin when neuronal cell lines are exposed to methamphetamine and cocaine. The application of siRNAs that block CREB phosphorylation also prevented ORF1 translocation to chromatin. This study demonstrates that methamphetamine and cocaine can serve as physiological stressors that impact the activity of TEs through various mechanisms. Doyle et al. (2017) found de novo LINE-1 insertions in postmortem dorsomedial prefrontal cortex from individuals who had been addicted to cocaine (Doyle et al. 2017). Many of these LINE-1 insertions were associated with genes involved with cocaine addiction, leading to the hypothesis that these insertions may predispose people to develop cocaine addiction.

5.2 Neurodevelopmental Disorders

Autism and Rett Syndrome In 2010, Muotri et al. (2010) were the first to show that LINE-1 introduces new insertions in neuronal progenitor cells (NPCs) during neurodevelopment (Muotri et al. 2010). The authors induced pluripotent stems cells (iPSC) from fibroblasts of children with Rett syndrome (RTT) with a particular frameshift mutation in the MeCP2 gene and observed increased LINE-1 retrotranposition compared to NPCs derived from a healthy control. Additionally, increased LINE-1 ORF1 expression was found in postmortem brain tissue taken from individuals with RTT relative to healthy controls.

Balestrieri et al. (2012) found increased expression of human ERV-H and reduced human ERV-W in the blood of children with autism spectrum disorder (ASD) relative to healthy controls (Balestrieri et al. 2012). Within people with ASD, elevated human ERV-H expression was significantly associated with severely impaired communication and motor skills, as measured by a psychometric test of these constructs. Extending research on TEs from peripheral tissue to central nervous system tissue, Shpyleva et al. (2018) found an increase in LINE-1 expression in postmortem cerebellar tissue taken from children with autism relative to healthy controls (Shpyleva et al. 2018). This increased expression was associated with reduced DNA repressive mechanisms (reduced histone H3K9me3 in ORF1 and ORF2, both of which are overexpressed in cerebellar tissue taken from individuals with autism). In this study, increased LINE-1 ORF1 and ORF2 expression was linked to increased oxidative stress (as indicated by a reduction in glutathione/GSSH

redox status), which is one potential trigger for LINE-1 retrotransposition (Shpyleva et al. 2018).

Attention Deficit Hyperactivity Disorder (ADHD) Relative to healthy controls, Balestrieri et al. (2014) found elevated expression of the envelope glycoprotein gene (*env*) of human ERV-H in blood samples from children with ADHD who had never taken any psychiatric medication, suggesting that there might be an inflammatory and/or neurotoxic component to ADHD that may be influenced by TE overexpression (Balestrieri et al. 2014). D'Agati et al. (2016) conducted a follow-up study in which an adolescent male with ADHD was treated with methylphenidate, a brain stimulant used to treat ADHD (D'Agati et al. 2016). Since methylphenidate increases availability of dopamine and norepinephrine in the brain, it is possible that this is the primary mechanism through which methylphenidate treats the symptoms of ADHD. However, it is also possible that methylphenidate impacts ADHD through inducing epigenetic changes that may or may not be related to neurotransmitter availability. After 6 months of treatment with methylphenidate, D'Agati and colleagues found a significant reduction in symptoms of ADHD and in human ERV-H env expression in this patient (D'Agati et al. 2016). Expression of human ERV-H env in this patient was compared to that in a small sample of healthy controls, and the patient's posttreatment level was below that of the healthy controls. This suggests that methylphenidate has the potential to strongly downregulate human ERV-H env expression. This downregulation could impact expression of genes typically associated with ADHD. It could potentially also lead to a reduction in inflammation and/or neurotoxicity, which could also be related to the reduction in symptoms.

5.3 Neurodegenerative Disorders

Alzheimer's Disease TEs transcriptional activity is usually under epigenetic control to monitor their potentially harmful reactivation. Several studies revealed that brain aging might lead to deteriorating TE surveillance. In Drosophila transgenic models of Alzheimer's disease (AD), this has been shown to lead to retrotransposon activation (Li et al. 2013; Maxwell et al. 2011; Wood et al. 2016). Despite the corroborating evidence from an established fly model relevant to AD, TE activation has not been extensively studied in humans up until very recently. A recent study examined TE profiles in 600 human cortical postmortem transcriptomes and found that TEs are substantially reactivated in AD patients' brains (Guo et al. 2018). The analysis of TE profiles in the postmortem dorsal lateral prefrontal cortex (DLPFC) of AD revealed that nine different types of TEs (regardless of their relative copy number or specific location within the genome), belonging to different classes and families, were significantly associated with the overall histologic count of protein Tau-derived neurofibrillary tangles, perhaps the most significant hallmarks of AD. This finding, corroborated by an experiment in a transgenic Drosophila model, leads to the hypothesis that chromatin relaxation induced by Tau protein

might be responsible for derepression of silenced TEs. Although further evidence should be accumulated, this is the first evidence shedding some light on the potential causal link between AD Tau pathology and TE reactivation in human brains.

Frontotemporal Dementia (FTLD) As highlighted in a study focusing on neurodegenerative disorders, there is a putative causal link between TE overexpression and TAR DNA-binding protein 43 (TDP-43), hnRNP-like RNA-binding protein implicated in numerous cellular functions including alternative splicing and regulation of mRNA stability. The accumulation of TDP-43 is a hallmark of several neurodegenerative disorders, such as AD and frontotemporal lobar degeneration (FTLD). The study, a meta-analysis of cross-linking immunoprecipitation sequencing (CLIP-seq) datasets in mouse and human investigating the RNA targets of TDP-43 protein, revealed a significant enrichment in target sequences that derive from many classes of TEs in healthy controls compared to FTLD patients (Li et al. 2012). These findings support a possible mechanism of TE silencing by which TDP-43 would normally function to regulate TE expression.

Ataxia Telangiectasia Ataxia telangiectasia is a primary immunodeficiency disease whose patient displays progressive neurodegeneration and eventual death in the second or third decade of their life. AT is a rare inherited disorder and is inherited as autosomal recessive pattern. The causal gene has been identified, ATM, a serine/threonine kinase that is a sensor of cellular DNA damage. Mutations that deactivate the gene result in AT. In 2011, Coufal and colleagues demonstrated an increase in LINE-1 retrotransposition in cells that contained reduced levels of the mutant copies of the ATM genes (Coufal et al. 2011). The researcher further observed an increase in LINE-1 copy number in postmortem brain tissue derived from AT patients. These findings supported the unprecedented hypothesis that LINE-1 might play a role in modulating the biological pathways revolving around repair of DNA damage.

X-Linked Dystonia Parkinsonism One of the few examples of disease involving a TE of the SVA family comes from the rare disorder of X-linked dystonia parkinsonism, a neurodegenerative movement disorder characterized by adult-onset parkinsonism and dystonia. Thanks to their sequence fidelity, SVA elements can dynamically affect genomic stability by facilitating inversions, duplications, and deletions leading the allelic imbalance (Hancks et al. 2011). As such, SVA elements are highly regulated by epigenetic factors (e.g., DNA methylation) which can affect the expression of neighboring genes. In the case of X-linked dystonia parkinsonism, SVA insertion and hypermethylation of the disease causal *TAF1* gene led to its reduced expression in the caudate (Makino et al. 2007).

6 Therapeutic Targeting of Transposable Elements

As reviewed more extensively above, TEs may have the potential to affect the expression of other genes through a variety of mechanisms. The ability of TEs to influence gene expression and protein production makes them potential targets for

drug development. Although the development of TE-associated drugs is still in its infancy, we review here, as proofs of principle, some examples derived from cancer and immune system disorders, in which this approach has already been successful. In contrast to single nucleotide or structural variation, reference TEs are shared by all patients, offering the advantage that they could be applied to entire patient populations.

The most experimentally interesting example is the therapeutic use of an antibody to target the expression of human ERV-W-Env in type 1 diabetes (T1D). Epidemiological studies revealed that pathogenicity is mediated by abnormally expressed human ERV-Env proteins in the blood and postmortem acinar pancreatic cells of T1D patients (Curtin et al. 2018). A monoclonal antibody (IgG4) has been developed to target and neutralize human ERV-W-Env in vitro and in vivo specifically. This pharmacological approach is currently under clinical development in T1D. If further stages of clinical efficacy will be successful, these results may lead to the development of innovative treatments for a disease associated with dysregulation of human ERVs expression.

Another example is the therapeutic use of inhibitors of reverse transcriptase (RT), the enzyme encoded as part of the open reading frame 2 of LINE-1 elements. RT has recently been proposed as a target for anticancer drugs, specifically prostate cancer. The mechanism involves its inhibition either via RNA interference-dependent signaling of active LINE-1 elements or using RT inhibitory drugs. As a result, the proliferation of cancer cells was significantly reduced, promoting their differentiation and antagonizing tumor progression in animal models (Houede et al. 2018; Sciamanna et al. 2016).

The effect of drugs that function as DNA methyltransferase (DNMT) inhibitors, generally used for epigenetic cancer therapy, on TEs is remarkable. These drugs are used to reactivate epigenetically silenced tumor suppressor genes. Unfortunately, not much is known about the sequence specificity of such a TE/tumor suppressor gene mechanism, and a possible emerging side effect could be the pharmacologically induced reactivation of epigenetically silenced TEs. A study revealed that the demethylation, and reactivation, of a LINE-1 antisense promoter, was able to drive the transcription of neighboring genes, even if the promoter of the gene itself was accurately silenced by the DNMT inhibitors (Weber et al. 2010). This example opens up a critical role of TEs in epigenetic therapy, whether in consideration of side effects or, hopefully in the future, of specific drug approaches designed on TE prerogative to provide alternative mechanisms to reactivate the transcription of particular genes.

These few examples prove that TEs may be experimentally pursued as potential therapeutic targets. We are only beginning to understand that there are many possible mechanisms for using TEs to manipulate gene expression and pathophysiological pathways. Once we can identify TEs involved in disease, their sequence could be used to design oligonucleotides that can inhibit or enhance the expression of a protein and modulate the symptoms of the disease, depending on the direction of the effect of the mechanism implicated.

7 Future Directions and Conclusions

The experimental evidence reviewed in previous sections of this chapter supports a role for TEs RNA-mediated epigenetic regulation and in the etiopathogenesis of neuropsychiatric diseases. TEs involved tend to include both long and short elements, with the most persuasive evidence pointing to insertional mutagenesis by LINE-1s and dysregulated transcriptional activity of HERVs. Much of the data available to date are still mostly anecdotal and merely descriptive. An ideal experiment would demonstrate the mechanism by which TEs mediate epigenetic effects and would show how these effects would be inhibited in the absence of the candidate TE. While such tests still present a lot of challenges, these are undoubtedly critical to unequivocally uncovering the functional mechanisms of TE regulatory function, and would allow the field to move forward. These data are especially relevant given the growing interest in the idea that the exposure to external factors, both biological and psychological stressors, can influence individuals' well-being and that TEs can mediate these processes. Few areas of biological science are more thought-provoking than the field of TEs. The underlying notion that TEs may be something other than silent "junk" DNA sequences runs counter to what was previously believed by scientists not long ago. Progress in this field now has the opportunity to advance quickly, almost as abundantly as controversies and debates. Preliminary evidence supports the increasing fascination with the many applications that arise from an increased understanding of TEs functional mechanisms.

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Multi-, Inter-, and Transgenerational Effects of Drugs of Abuse on Behavior

Fair M. Vassoler, Anika M. Toorie, and Elizabeth M. Byrnes

Contents

Abstract Transgenerational epigenetic inheritance is a burgeoning field that has recently garnered much attention. A growing body of evidence identifies behavioral phenotypes associated with inter-, multi-, and transgenerational studies following a wide variety of parental exposures. This chapter in current topics in behavioral neurogenomics examines the evidence for the presence of behavioral phenotypes and, in particular, the varied and often opposite behavioral responses observed with protocol shifts. Effects following parental exposure to drugs of abuse are used as an example of the wide range of behavioral outcomes and the variability associated with these multiple generation studies. The behavioral phenotypes associated with drug exposure are reviewed in depth.

Keywords Addiction · Behavior · Drugs of abuse · Epigenetic · Transgenerational

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1 Introduction

Historically, the prevailing hypothesis has been that phenotype is determined largely by genotype, with the acknowledgement that some aspects of an individual's phenotype may be further influenced by environmental factors. Thus, both nature (genetics) and nurture (environmental experiences) play a role in phenotypic variations observed in both human and animal populations. A growing body of evidence, however, suggests that phenotypic variation may also be shaped by parental experiences occurring prior to conception, which are then transmitted to offspring in the absence of alterations in DNA sequence. This phenomenon has been termed *epigenetic inheritance*. Because the term epigenetics is used mechanistically to describe a multitude of specific genomic events, it is worth devoting attention to a precise and careful definition that will be used in the current chapter.

The term epigenetics was first coined by Conrad Hal Waddington in 1942 to describe the relationship between genotype and the embryonic environment, which together determine cellular differentiation, and ultimately, phenotype. Importantly, most definitions of epigenetics include some factor of heritability, whether it be across cell divisions or across generations. Perhaps one of the most well-cited definitions is that of Adrian Bird, who in 2007 wrote, "The following could be a unifying definition of epigenetic events: the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states." This definition encompasses chromosomal marks and includes both stable changes maintained across multiple cell generations and changes inherited in offspring. For the purposes of this chapter, we will utilize a relatively conservative definition that defines epigenetics as *the perpetuation of specific gene activation states resulting in phenotypic variations that persist across generations in the absence of changes to the DNA sequence*.

Within the burgeoning field of epigenetic inheritance, another important distinction must be recognized, that of inter-, multi-, and transgenerational phenomena. This definition relates to the nature of the exposure (direct versus indirect) experienced by a particular generation (Daxinger and Whitelaw 2012; Ferguson-Smith and Patti 2011; Heard and Martienssen 2014; Lim and Brunet 2013; Nagy and Turecki 2015). The terms inter- and multigenerational refer to studies that involve an environmental stimulus applied to the parental generation (F0 or P0) and the resulting phenotypic variations observed in the offspring. Intergenerational studies examine first-generation offspring (F1) following a parental manipulation; however, because the germ cells that produce the F1 generation are present during the manipulation, F1 animals are considered to have experienced a direct exposure. Such studies are deemed intergenerational, i.e., they span two generations with direct exposure. A multigenerational design often spans more than two generations but still suggests direct exposure across all generations examined. For example, during in utero manipulations, the developing fetus is directly exposed to the environmental manipulation (F1 direct exposure). The second generation (F2), however, also has a direct exposure as the developing fetus already contains the germ cells that will

become the F2 animals. This is a classic example of a multigenerational study (spans multiple generations all with direct exposure). Traditionally, to be considered a transgenerational study, at least one generation is examined that never received any direct exposure. A clear definition, proposed by Skinner in 2011, states that transgenerational epigenetic inheritance is "germline-mediated inheritance of epigenetic information between generations in the absence of direct environmental influences that leads to phenotypic variation" (Skinner 2011). Thus, this is a distinct phenomenon because it implies that there is some stable change in the epigenome of the animal that is inherited beyond any generation that experienced direct exposure.

There is a growing body of evidence that shows a wide range of behavioral, molecular, and physiological phenotypes in inter-, multi-, and transgenerational studies. Interestingly, the phenotypes often vary widely even within similar exposure protocols. It is the goal of this chapter to detail inter-, multi-, and transgenerational behavioral endpoints following parental exposures. Given the breadth of current findings, we will utilize the behavioral endpoints following parental exposure to drugs of abuse as an example of the varying and complex behavioral outcomes in order to highlight some of the challenges facing researchers. In fact, skepticism remains in the field due to difficulties with conducting these complex experiments (Martos et al. 2015; Nagy and Turecki 2015). Moreover, variation observed between different models (rat versus mouse models) also serves to demonstrate the wide variability in outcomes with these studies and underscore the uniqueness of this phenomenon in different species. Both prenatal studies and exposure-specific reviews have recently arisen, so the goal of this chapter will be to define appropriate next steps for this burgeoning field.

For reviews of prenatal studies, see Behnke and Eyler (1993), Bock et al. (2015), Graignic-Philippe et al. (2014), Rohan et al. (2011), Sithisarn et al. (2012), and Veru et al. (2014).

For a review of behavioral phenotypes following different exposures, see Babenko et al. (2015), Chastain and Sarkar (2017), Park et al. (2017), Skinner (2014), Vassoler et al. (2014), and Yuan et al. (2016).

2 Behavioral Outcomes Following Parental Exposure to Drugs of Abuse

One of the most studied phenomena in the field remains that of consequences of exposure to drugs of abuse on subsequent generations. This is particularly compelling because of the high rates of recreational use, prescriptions, misuse, and abuse of drugs by humans. In terms of behavioral effects that have been tested, they can be broadly divided into four categories: effects on locomotor activity, affective behaviors, learning and memory/attention, and effects on reward-related behaviors.

2.1 Changes in Basal Activity Level Following F0 Drug Exposure

The monitoring of locomotor activity in rodents is one of the most common methods to track and screen for generalized changes in behavior. Locomotor activity studied under specific environmental conditions can differentiate behavioral outcomes. Specific categories are often investigated, including measures of general hypo- or hyperactivity, anxiety-like behavior, motivated exploration, and highly repetitive sequences of locomotor behavior (i.e., stereotypy). The focus on locomotor behavior in the context of drug exposure also demonstrates translational value, with evidence from human studies suggesting an association between exposure to drugs of abuse and alteration in locomotor activity in children. For example, there is a correlation between parental alcoholism and offspring childhood hyperactivity (Goodwin et al. 1975). Therefore, offspring locomotor behavior is often measured when examining the phenotypic differences in multi-, inter-, and transgenerational studies.

A number of studies in rodents have examined the effects of parental alcohol consumption on locomotor activity in offspring. In one such study, male rats were intubated twice daily with alcohol for 9 weeks and then bred with untreated females. Controls included pair-fed and vehicle-intubated males, as well as non-treated ad-lib-fed males. Locomotor activity in subsequent offspring was tested at 21, 42, and 90 days of age. Increased locomotor activity across all ages was associated with paternal alcohol consumption, and these effects were mediated, at least in part, by changes within the cholinergic system, which suggests alterations that could affect many aspects of behavior including learning and memory as well as attention (Abel 1993, 1994). Another study, however, observed decreased activity associated with paternal alcohol consumption in rats (Abel 1989b). Indeed, it appears that effects on locomotor activity are dependent upon both the duration of drinking prior to insemination and the age of the offspring at the time of testing (Abel 1989a). In addition to basal locomotor activity, psychostimulant-induced hyperlocomotion has also been examined following paternal alcohol consumption. Dose-dependent effects on amphetamine-induced locomotor behavior in the offspring of alcohol-exposed males have been observed, with increased locomotor activity observed following high doses and blunted locomotor activity following low doses (Abel 1993). Moreover, if the paternal alcohol consumption is voluntary (e.g., in the drinking water), there is a dose-dependent decrease in locomotor activity on post-natal days (PND) 20 and 24 (Abel and Lee 1988) accompanied by a dose-related decreased in testosterone at PND 55 (Abel 1989b; Abel and Lee 1988). It should be noted that these studies on voluntary intake were conducted in mice, while all of the other aforementioned studies were performed in rats. Overall, however, these studies suggest that divergent effects on locomotor behavior may be dependent on the duration of exposure, on the age at testing, and perhaps on whether alcohol exposure is forced or voluntary.

Alcohol is not the only drug that affects offspring locomotor behavior. Paternal cocaine exposure also results in hyperlocomotion in the offspring. For example,

cocaine administered to male Long Evans rats for a minimum of 72 days results in offspring hyperactivity (Abel 1989b). This effect, however, was not observed when examined using a mouse model (Killinger et al. 2012), suggesting that species and perhaps strain can influence such intergenerational effects. Indeed, strain differences in behavioral response to drugs of abuse have been demonstrated (Cabib et al. 2000), and therefore it would not be surprising if strain interacted with environment to produce different generational effects. Interestingly, prenatal nicotine exposure in female mice causes hyperlocomotion in F2 and F3 offspring of the maternal but not paternal lineage (Zhu et al. 2014). Taken together, studies performed to date demonstrate both intergenerational and transgenerational effects on locomotor activity following parental drug exposure. The specific direction and magnitude of the effect are dependent on numerous factors, including the sex of the parent, the age of the offspring at testing, the type and duration of the exposure regimen, the drug, as well as the species/strain examined. Such wide variations are not unexpected when one considers that at its essence epigenetic inheritance involves interplay between genotype, embryological milieu, and environmental factors throughout development.

2.2 Changes in Affective Behaviors Following F0 Drug Exposure

Substance use disorders and mood disturbances are highly correlated with significant comorbidity. For example, compared with the general population, individuals with a substance use disorder are more than twice as likely to suffer from a mood and/or anxiety disorder; and the reverse is also true (Geller 1988; Hasin and Kilcoyne 2012; Hasin and Grant 1987; Pasche 2012; Wu and Blazer 2014). Such findings suggest significant interaction between the neural mechanisms underlying substance abuse and affective disorders. Based on this interrelatedness, measures of anxiety- and depressive-like behaviors in rodents are often examined following F0 drug exposure. These studies have revealed significant epigenetic effects as a function of parental drug exposure.

With regard to alcohol, paternal alcohol consumption leads to decreased immobility in the forced swim test in the offspring, which has been interpreted as reduced depressive-like behavior (Abel 1991a). However, such findings may be confounded by the generalized alterations in locomotor behavior detailed previously. Moreover, rat offspring of alcohol-consuming males also demonstrate decreased grooming, which may suggest a blunted response to environmental stimuli, which is often associated with a depressive-like phenotype (Abel 1991b). Similar to locomotor effects, rats and mice seem to have opposite effects in the forced swim test following paternal alcohol consumption. Mice offspring sired by alcohol-consuming males had increased immobility on the FST, which was reversed by imipramine (Abel and Bilitzke 1990). Of note, this same study included rats and replicated the previous

forced swim test finding of decreased immobility, providing additional support for significant species differences on the intergenerational effects of paternal alcohol (Abel and Bilitzke 1990).

Even very limited exposures to alcohol prior to mating have been shown to affect subsequent generations. Thus, males administered acute alcohol just prior to mating sired pups that had delays in meeting developmental milestones in comparison to controls. They showed delayed surface righting, clinging, tail-pull reflex, rotation, linear movement, and climbing an inclined surface (Meek et al. 2007). As adults, the alcohol-sired group showed blunted risk assessment behavior, worse attention, and decreased anxiety-like behavior as measured with defensive probe burying (Meek et al. 2007). They were also more aggressive in social interactions (Meek et al. 2007). Together, the results indicate that acute alcohol administered just prior to mating causes offspring with delayed physical development, increased aggression, and decreased fearfulness (Meek et al. 2007). The mechanisms involved in the transmission of these effects remain unknown.

Opioid exposure in the F0 generation has also been shown to affect mood-related behaviors in subsequent generations. In one model, adolescent females are administered morphine or saline for 10 days during adolescence and then bred with naïve males after a 3–4-week drug-free period. Differences in their offspring are apparent even during early postnatal development, with F1 offspring demonstrating decreased maternal separation-evoked ultrasonic vocalizations (Bodi et al. 2016). During adulthood, F1 rats show increased time spent in the center of an open field indicating reduced anxiety-like behavior, although this effect was only observed in females during diestrus (Byrnes et al. 2011). In contrast, there was increased anxiety-like behavior as measured in the elevated plus maze (less time on the open arms) and decreased novel environment exploration in the offspring of adolescently morphine-exposed females compared to saline controls (Byrnes 2005). In addition, adolescent F1 males demonstrate decreased rough and tumble play behavior, while F1 females show increased play behaviors (Johnson et al. 2011). This effect seems to suggest convergence of gender-typical social behaviors, which could have significant implications over time.

Paternal cocaine exposure in a mouse model did not affect anxiety-like behavior in the offspring yet increased immobility in the tail suspension test. As tail suspension is used as a measure of depressive-like behavior, such findings suggest an increase in depressive-like behaviors (Killinger et al. 2012). However, another study reported the opposite outcome, i.e., male offspring of cocaine-exposed males demonstrated increased anxiety-like behavior with no change in depressive-like behavior (Fischer et al. 2017). This second data set matches results from rats, using a model in which males were allowed to self-administer cocaine for an extended period during adulthood prior to siring offspring. In that study, there was increased anxiety-like behavior in the male offspring as measured by novelty-induced hyperphagia paradigm and defensive probe burying task (White et al. 2016). Although there was no effect on depressive-like behavior as measured by forced swim task (White et al. 2016), once again, there seem to be divergent effects depending on the animal model, exposure regimen, and age at exposure and testing. These discrepancies

actually serve to reconcile the divergent literature showing a wide range of behavioral phenotypes in multi-, inter-, and transgenerational models.

2.3 *Changes in Learning and Memory and Attention Following F0 Drug Exposure*

Substance use disorders are sometimes thought of as a dysfunction of the learning and memory system. Drug taking causes a pathological subversion of normal brain learning such that the motivation to seek and take a drug as well as stimuli associated with drug intake leads to compulsive use (Robbins and Everitt 2002). In addition, there is some evidence that drug use itself disrupts normal learning, memory, and attention processes (Taubenfeld et al. 2010; Torregrossa et al. 2011). Thus, these same processes are examined in the offspring in order to determine if there is any transmission of these acquired deficiencies. Indeed, multiple drug models have observed deficits in learning, memory, and/or attention in subsequent generations.

Male rats intubated with alcohol produce offspring that have delays in learning a passive avoidance task during early adolescence (Abel 1994). In rats that were allowed to freely consume alcohol (as opposed to oral gavage), female offspring showed deficits in learning a two-way shock avoidance task, although there were no differences in passive avoidance or spontaneous alternation (Abel and Tan 1988). Extended paternal alcohol consumption that ceased 2 weeks prior to conception produced offspring that did not differ on developmental milestones or locomotor activity nor on object exploration and recognition tasks (Wozniak et al. 1991). However, male offspring did demonstrate impaired acquisition of a spatial learning discrimination task utilizing either a radial arm maze or a T-maze (Wozniak et al. 1991). In contrast, in mice, high doses of paternal alcohol produced offspring requiring fewer trials to learn a passive avoidance task which was associated with longer latencies to reach a choice point in a T-maze (Abel and Lee 1988). Paternal preconception ethanol in mice also produces F1 offspring with attention deficits such as inattention and increased impulsivity (Kim et al. 2014).

Paternal cocaine exposure also results in F1 offspring with memory deficits. Extended adult cocaine self-administration during adulthood causes deficits in novel place recognition task in male offspring but not novel object recognition (Wimmer et al. 2017). Similarly, with paternal cocaine exposure in mice, the offspring have deficits in sustained visuospatial attention and spatial working memory utilizing the 5-arm maze paradigm (He et al. 2006). There was also a decreased percentage of correct trials at the shortest stimulus duration in male F1 as well as deficits in working memory (He et al. 2006). The deficit was worse in females appearing at longer stimulus durations and shorter intertrial intervals (He et al. 2006).

2.4 *Altered Responsivity to Drugs of Abuse Following F0 Drug Exposure*

Substance use disorders run in families, yet a genetic component that completely explains the heredity has yet to be determined. There have been many genome-wide association studies that have searched for genetic components to explain inheritance patterns of addiction, and the lack of any definitive gene or set of genes has been termed "missing heritability." It has been proposed that transgenerational epigenetic inheritance may play a role in this missing heritability. It is not surprising then to find that considerable work on F0 exposure to drugs of abuse has focused on alterations in the reward system and offspring susceptibility to addiction-like disorders.

Based on clinical observations, it was widely predicted that increased sensitivity to drugs of abuse would be observed in future generations. There is some evidence to support this hypothesis. For example, in rats, paternal alcohol exposure caused increased locomotor response to amphetamine in F1 offspring (Abel 1993). This could be interpreted as a cross-sensitization phenotype of the F1 animals. Cross-sensitization is a phenomenon whereby sensitization to a particular stimulus (such as a drug of abuse) is generalized to a related stimulus (a different drug of abuse), which results in the amplification of a response to both the original and the related stimulus. Therefore, the presence of an increased response to amphetamine may indicate an increase in the addiction liability in those animals. Some of the most compelling evidence for increased susceptibility to addiction-like disorders in offspring arises from adolescent cannabinoid exposure studies. For example, it was shown that adolescent cannabinoid exposure to the synthetic cannabinoid WIN-55,212 induces an enhanced sensitivity to morphine-induced conditioned place preference in male F1 offspring (Byrnes et al. 2012). In addition, female offspring demonstrate an enhanced expression of morphine-induced locomotor sensitization (Vassoler et al. 2013a). It was later shown that adolescent THC exposure (the psychoactive cannabinoid found in marijuana) in both parents increases the effort that offspring will work toward heroin self-administration (Szutorisz et al. 2014). Cocaine exposure in females prior to pregnancy enhances the acute locomotor response to cocaine in adult male offspring (Sasaki et al. 2014). Finally, intergenerational effects on drug sensitivity have also been shown with adolescent morphine exposure. Female adolescent morphine exposure causes a more rapid induction of morphine-induced locomotor sensitization in female offspring and a significant enhancement of sensitization in male offspring (Byrnes 2005). Moreover, increased sensitivity to the analgesic effects of morphine and more rapid development of tolerance have also been observed in these F1 offspring (Byrnes et al. 2011). Lastly, there were increases in the preference for lower doses of morphine as measured by conditioned place preference (CPP; Vassoler et al. 2016).

Yet, for each of these classes of drugs and exposure regimens, there has been an equally large (if not larger) body of evidence that suggests a protective effect in the offspring where exposures in parental generations seem to be preparing offspring for similar environments. This hypothesis, termed the "fetal origins of adult disease

(FOAD) hypothesis," has been gaining supporting evidence from multiple fields (Calkins and Devaskar 2011). Thus, paternal alcohol exposure reduces ethanol preference and consumption in adult male offspring yet increases sensitivity to the anxiolytic and motor-enhancing effects of ethanol (Finegersh and Homanics 2014). Adolescent female cannabinoid exposure diminishes the reward-facilitating effects of low doses of THC as well as amphetamine in adult male offspring as measured using intracranial self-stimulation (Pitsilis et al. 2017). Female adolescent morphine exposure attenuates quinpirole-induced locomotor sensitization in F1 and F2 male offspring, demonstrating transgenerational reductions in sensitivity to the dopaminergic system (Byrnes et al. 2013). Indeed, when examining self-administration, there are a decrease in self-administration acquisition across all doses tested in females and a particularly striking blunting of morphine-induced reinstatement in males that extends to the F1 and F2 generations, another transgenerational effect (Vassoler et al. 2017). In one study where fathers were allowed to binge-eat high-sucrose foods, cocaine- and sucrose-seeking behaviors were blunted in the offspring (Le et al. 2017a). The offspring also showed decreases in reward seeking and increases in anxiety-like behavior (Le et al. 2017a).

Further evidence in support of the FOAD hypothesis stems from paternal cocaine work. In mice, paternal cocaine injections for 75 days produced female offspring with blunted cocaine preference (Fischer et al. 2017). Additionally, females and male F1 s exhibited increased sensitivity to the psychomotor effects of cocaine and amphetamine (Fischer et al. 2017). This point reconciles some of the disparate data as it appears that reward and locomotor response are not necessarily correlated. Therefore, although animals may demonstrate increased locomotor response to particular drugs of abuse, their reward-related response may be blunted. F1 animals sired by cocaine-exposed males also consumed more sucrose, demonstrating specific alterations in reward pathways (Fischer et al. 2017).

In a study where male rats were trained to self-administer cocaine for 60 days during adulthood, 24 h prior to mating with naïve females, it was found that male F1 offspring demonstrated decreased acquisition of cocaine self-administration and reduced effort in a progressive ratio schedule (Vassoler et al. 2013b). Intriguingly, this study was then replicated by an independent group, and it was further discovered that if the sires are separated into addiction-like groups and non-addiction-like groups, the F1 and F2 offspring of high-responding sires had increased incentive response to cocaine (Le et al. 2017b). Thus, when all animals are examined together, there is a general trend toward decreased self-administration in the offspring, but when the animals are separated based on response, the highly drug-motivated sires transmit this phenotype to their offspring and grand offspring (Le et al. 2017b). This is particularly interesting when the human substance use patterns are taken into consideration. The majority of the population can consume rewarding substances recreationally and not develop a substance use disorder. The minority that does develop a substance use disorder may have additional genetic and epigenetic changes that prime them for the disease.

Taken together these reviewed studies demonstrate the breadth and variability of behavioral phenotypes in multi-, inter-, and transgenerational studies. Moreover, it is

clear that relatively minor exposures result in long-term and transgenerational behavioral phenotypes but also fairly minor modifications to protocols result in divergent and sometimes opposite responses. While some skepticism exists about the mechanism of action of transfer between generations, it is clear that environmental exposures can interact with genetics to alter behavior in subsequent generations.

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Correction to: Role of MicroRNAs in Anxiety and Anxiety-Related Disorders

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