Chapter 17
Epigenetic Mechanisms of Perinatal Programming: Translational Approaches from Rodent to Human and Back

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Abstract Perinatal life is a period of enhanced plasticity and susceptibility to environmental effects via the maternal environment or parental care. A variety of studies have indicated that epigenetic mechanisms, which can alter gene function without a change in gene sequence, play a role in setting developmental trajectories that impact health, including mental health. This chapter reviews examples of translational approaches to the study of biological embedding of mental health via differences in parental care.

17.1 Introduction

A prominent feature of parent care effects on mental health is its influence on the hypothalamic–pituitary axis, a major endocrine regulator of the response to psychosocial stress. Laboratory rodent models have been particularly useful in identifying mechanisms of epigenetic regulation in the brain that have then been used to generate hypotheses in humans. At the same time, recent advances in genomics have provided new means to address these questions in large numbers of human subjects in an increasingly comprehensive and powerful manner.

17.1.1 How Mechanisms of Gene Regulation "Above the Genome" Contribute to Interindividual Differences in Behavior

The advent of new high-throughput DNA sequencing technologies, initiated near the turn of the last century, has allowed the elucidation of the genetic sequence identities of humans and a growing list of other species. Where human health is concerned, these technologies were thought to herald a complete understanding of

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biological diversity, with strong implications for understanding the ultimate origins of behavior and psychopathology. However, this exuberance turned out to be misplaced. Our current understanding of genome function indicates that the DNA code must be exquisitely programmed by molecular mechanisms "above the genome," termed epigenetic. At a fundamental level, these mechanisms are now known to be crucial for conferring cell-type specificity during development by programming unique patterns of gene expression among the body's 200+ cell types. It is also now known that cell-type-specific patterns of epigenetic modifications are not exclusively genetically determined, and are to some degree responsive to environmental signaling throughout life. As we discuss below with specific examples, there is accumulating evidence indicating that phenotypic variation observed in humans and animal models can result from changes in gene function via epigenetic changes. The dynamic nature of epigenetic signaling contrasts with the static nature of the genetic code, and provides a mechanism of gene × environment interactions that bridge inherited variation with variation originating from environmental and stochastic sources. It should be noted, however, that these sources of variation are not necessarily mutually exclusive, as genetic variation can interact with epigenetic variation. However, in contrast to the static nature of genetic variation, epigenetic variation is potentially amenable to environmental or therapeutic intervention. Thus, we and others have argued that the study origins of health and human disease—including psychiatric disorders—is incomplete without an understanding of genetic, environmental and stochastic contributions to epigenetic signaling (Petronis 2010; Sasaki et al. 2013).

17.1.2 What Is "Epigenetics"?

We have alluded to epigenetic mechanisms as those that change gene function in the absence of a change in DNA sequence. It should be noted that the definition of the term "epigenetics" continues to be a matter of debate. The historical definition emphasized the heritability of changes in gene function without a change in gene sequence. This definition implied that such changes were trans-generational, passing from the maternal environment to the offspring via gametic inheritance. However, it is clear that such a definition leaves out important changes in gene function that do not involve changes in gene sequence, yet are relatively stable and passed from cell to daughter cell during mitosis. It also leaves out mechanisms that program changes in gene function in postmitotic cells such as neurons, heart cells, and other cells that do not replicate and would thus be left out of the epigenetic sphere. It may be equally unwise, however, to characterize all changes of gene function in the absence of a change in gene sequence as "epigenetic." The firing of neurons is a process that initiates a change in the expression of hundreds of genes, yet many of these are distinct from processes that result in the long-term potentiation of neural responses. Clearly, extending the definition of epigenetics to encompass mechanisms altering gene expression outside the realm of the DNA sequence requires careful consider-

ation of the *outcomes* associated with the epigenetic change. We and others have proposed a broad definition of epigenetics that includes long-term changes in gene function that are meiotically or mitotically heritable (Sasaki et al. 2013).

17.1.3 Gene Sequence and Its Relationship to Epigenetic Changes

The pattern of gene expression in each cell type confers its tissue-specific phenotype. As a result, a change in gene function as a result of a sequence alteration could alter the structure of the protein encoded by the gene or its activity by interfering with factors that increase or decrease its activity. Sequence variation has been associated with behavioral pathologies but with the exception of a minority of psychopathology linked to Mendelian disorders, such variation is inevitably linked to specific environmental conditions and thus is very rarely predicted solely on the basis of genetic variance. This has created something of a crisis in psychiatric research and a search by some for intermediary mechanisms that might help explain a missing heritability not directly attributable to genetic variance (Petronis 2010). In fact, it has been known for some time that epigenetic and genetic variation can predict the same biological outcomes.

We propose that understanding epigenetic mechanisms associated with psychopathology is important for at least three reasons: (1) Identifying alterations in epigenetic signaling associated with psychopathology may help explain intermediate mechanisms between the genome and stochastic and environmental factors involved in behavioral pathology; (2) defining these relationships could provide a biological understanding of gene × environment interactions; (3) because epigenetic mechanisms are potentially reversible, we may be able to identify novel therapeutic interventions to prevent or reverse these changes. In what follows, we provide a synopsis of our work addressing stable epigenetic changes associated with early-life environment in humans and rodent models. To do so, we first describe known molecular epigenetic processes that are best understood as mediators of these effects.

17.2 The Epigenome

17.2.1 Chromatin and the Histone Code

The epigenome consists of the chromatin and its modifications as well as a covalent modification by methylation of cytosine rings found at the dinucleotide sequence CG (Razin 1998). The epigenome determines the accessibility of the transcription machinery, which transcribes the genes into messenger RNA, to the DNA. Inaccessible genes are therefore silent whereas accessible genes are transcribed. We therefore distinguish between open and closed configuration of chromatin (Groudine

et al. 1983; Marks et al. 1985; Ramain et al. 1986; Grunstein 1997; Varga-Weisz and Becker 2006). Densely packaged chromatin can be visualized microscopically and is termed heterochromatin while open accessible chromatin is termed euchromatin. Another level of epigenetic regulation by small noncoding RNAs termed microRNA (miRNA) has been discovered (Bergmann and Lane 2003). miRNAs regulate gene expression at different levels; silencing of chromatin, degradation of mRNA, and blocking translation. miRNAs have been linked to behavioral pathologies in humans and regulate gene function through a variety of mechanisms, as has been extensively reviewed elsewhere (Vo et al. 2005; Mehler and Mattick 2006, 2007; Qureshi and Mehler 2009). These mechanisms are currently the subject of intense investigation. miRNA expression in adulthood has been linked to early-life stress in rats (Uchida et al. 2010) and almost certainly plays a more important role than is currently understood.

The DNA is wrapped around a protein-based structure termed chromatin. Chromatin is formed by an octamer of histone proteins termed a nucleosome. Variants of histone proteins, H1, H2A, H3B, H3, and H4 (Finch et al. 1977) and other minor variants have specific functions in DNA repair and gene activity (Sarma and Reinberg 2005). The octamer structure of the nucleosome is composed of a H3–H4 tetramer flanked on either side with a H2A–H2B dimer (Finch et al. 1977). The N terminal tails of these histones are extensively modified by methylation (Jenuwein 2001), phosphorylation, acetylation (Wade et al. 1997), and ubiquitination (Shilatifard 2006). The state of modification of these tails plays an important role in defining the accessibility of the DNA. The amino terminal tails of H3 and H4 histones that are positively charged form tight interactions with the negatively charged DNA backbone, thus blocking the interaction of transcription factors with the DNA. Modifications of the tails neutralize the charge on the tails, thus relaxing the tight grip of the histone tails. Different histone variants, which replace the standard isoforms also play a regulatory role and serve to mark active genes in some instances (Henikoff et al. 2004). The specific pattern of histone modifications was proposed to form a "histone code" that delineates the parts of the genome to be expressed at a given point in time in a given cell type (Jenuwein and Allis 2001). A change in histone modifications around a gene will change its level of expression and could convert a gene from an active state to a silent state, resulting in "loss of function" or switch a gene from a silent state to an active state, leading to "gain of function."

17.2.2 Histone-Modifying Enzymes and Chromatin Remodeling

The most-investigated histone-modifying enzymes are histone acetyltransferases (HAT), which acetylate histone H3 at the K9 residue as well as other residues and H4 tails at a number of residues, and histone deacetylases (HDAC), that deacetylate histone tails (Kuo and Allis 1998). Histone acetylation is believed to be a predominant signal for an active chromatin configuration (Perry and Chalkley 1982; Lee et al. 1993). Deacetylated histones signal inactive chromatin, associated with

inactive genes. Many repressors and repressor complexes recruit HDACs to genes, thus leading to their inactivation (Wolffe 1996). Histone tail acetylation is believed to enhance the accessibility of a gene to the transcription machinery whereas deacetylated tails are highly charged and believed to be tightly associated with the DNA backbone and thus limiting accessibility of genes to transcription factors (Kuo and Allis 1998).

Histone modification by methylation is catalyzed by different histone methyltransferases. Some specific methylation events are associated with gene silencing and some with gene activation (Lachner et al. 2001). Particular factors recognize histone modifications and further stabilize an inactive state. For example, the heterochromatin-associated protein HP-1 binds H3-histone tails methylated at the K9 residue and precipitates an inactive chromatin structure (Lachner et al. 2001). Recently described histone demethylases remove the methylation mark causing either activation or repression of gene expression (Shi et al. 2004; Tsukada et al. 2006).

Chromatin remodeling complexes, which are ATP dependent, alter the position of nucleosomes around the transcription initiation site and define its accessibility to the transcription machinery (Varga-Weisz and Becker 2006). It is becoming clear now that there is an interrelationship between chromatin modification and chromatin remodeling. For example, active regions of the chromatin are associated with hypomethylated DNA, and hypermethylated DNA is packaged in inactive chromatin (Razin 1998; Razin and Cedar 1977).

17.2.3 Targeting of Chromatin-Modifying Enzymes to Specific Genes

To date, there are relatively few examples from neuroscience research of gene targeting of epigenetic mechanisms, though we review one such example below for maternal care. In our view, this is a fundamental principle of epigenetic regulation of gene expression that will shed important light on neuronal gene regulation. There are, however, many examples of targeting from other areas of research, a few of which we describe below as illustrative examples. Transcription factors and repressors recruit the nonspecific histone-modifying enzymes to specific genomic loci and target-specific genes (Jenuwein and Allis 2001). Transcription factors and repressors recognize specific *cis*-acing sequences in genes, bind to these sequences, and attract specific chromatin-modifying enzymes to genes through protein–protein interactions. Specific transacting factors are responsive to cellular signaling pathways. Signal transduction pathways are activated by cell surface receptors and could thus serve as conduits for epigenetic change linking the environmental trigger at cell surface receptors with gene-specific chromatin alterations and modulation of programming of gene activity. For example, numerous signaling pathways including those triggered by G-protein-coupled cell surface receptors in the brain alter the concentration of cyclic adenosine monophosphate (cAMP) in the cell. One of the transcription factors which respond to increased cAMP is cAMP response

element-binding protein (CREB). CREB binds cAMP response elements in certain genes. CREB also recruits CREB-binding protein CBP. CBP is a HAT, which acetylates histones (Ogryzko et al. 1996). Thus, elevation of cAMP levels in response to an extracellular signal would result in a change in the state of histone acetylation in specific genes. Environmental or physiological events that interfere at any point along the signaling pathway may result in chromatin alterations. Below, we discuss an example of such a pathway that leads from maternal behavior to long-term programming of gene expression in the hippocampus (Meaney and Szyf 2005).

17.2.4 DNA Methylation

In addition to chromatin, which is associated with DNA, the DNA molecule itself is chemically modified by methyl residues at the 5′ position of the cytosine rings in the dinucleotide sequence CG in vertebrates (Razin 1998). Other modifications to DNA, including hydroxymethylation (5-hmC) and several other DNA modifications, are attracting increasing interest as potential gene regulatory mechanisms (Labrie et al. 2012). It should be noted that conventional methods used for mapping 5-mC, such as bisulfite sequencing and methylation-sensitive restriction enzyme-based approaches, do not differentiate it from 5-hmC, although it is possible to use enzyme-based glycosylation of 5-hmC followed by restriction enzyme-based detection of 5-hmC and 5-mC or as via modified bisulfite sequencing (Booth et al. 2012). In this chapter, we use the term "DNA methylation" to denote epigenetic changes associated with the DNA itself, though the term "DNA modification" is perhaps more accurate given the current knowledge.

Among different cell types, distinct CG methylation generates cell-type-specific epigenetic patterns. Thus, the DNA methylation pattern confers upon the genome its cell-type identity (Razin 1998). The DNA methylation pattern is established during development and is then maintained throughout life by the maintenance DNA methyltransferases (DNMT; Razin and Riggs 1980). DNA methylation in distinct regulatory regions is believed to generally mark silent genes. Thus, aberrant methylation will silence a gene, resulting in "loss of function," which will have a similar consequence as a loss of function by genetic mechanism such as mutation, deletion, or rearrangement.

17.2.5 DNA Methylation Enzymes

The DNA methylation pattern is not copied by the DNA replication machinery, but by independent enzymatic machinery the DNMT (Razin and Cedar 1977). The DNA methylation machinery in vertebrates has two main roles. First, it establishes new cell-type-specific DNA methylation patterns during development and possibly during adulthood in response to new signals. Second, it maintains these patterns during downstream cell divisions and after DNA repair. The different enzymes and

proteins of the DNA methylation machinery must address these different tasks. The methylation of DNA occurs immediately after replication by a transfer of a methyl moiety from the donor *S*-adenosyl-L-methionine (AdoMet) in a reaction catalyzed by DNMTs. The maintenance DNMT1 prefers a hemimethylated substrate (Razin and Riggs 1980). Since hemimethylated sites are generated during DNA replication when a nascent unmethylated C is synthesized across a methylated C in the template parental strand, the DNMT accurately copies the methylation pattern of the template strand. Three distinct phylogenic DNMT were identified in mammals. DNMT1 shows preference for hemimethylated DNA in vitro, which is consistent with its role as a maintenance DNMT, whereas DNMT3a and DNMT3b methylate unmethylated and methylated DNA at an equal rate which is consistent with a de novo DNMT role (Okano et al. 1998). Knockout mouse data indicate that DNMT1 is responsible for a majority of DNA methylation marks in the mouse genome (Li et al. 1992) whereas DNMT3a and DNMT3b are responsible for some but not all de novo methylation during development (Okano et al. 1999).

The answer to the question of whether the DNA methylation is reversible or not has important implications on the possibility that DNA methylation is dynamic and responsive to physiological and environmental signals throughout life. DNMTs are present in neurons (Goto et al. 1994) and there are data suggesting that DNMT levels in neurons change in certain pathological conditions such as schizophrenia (Veldic et al. 2005). The presence of DNMT in neurons suggests that DNA methylation is dynamic in postmitotic tissues and is a balance of methylation and demethylation reactions (Szyf 2001).

17.2.6 DNA Demethylation Enzymes

We and others have proposed that the DNA methylation pattern is a balance of methylation and demethylation reactions that are responsive to physiological and environmental signals and thus serves as a biological manifestation of gene–environment interactions (Sasaki et al. 2013; Szyf et al. 2007; McGowan and Szyf 2010). There are now convincing examples of active, replication-independent DNA demethylation during development as well as in somatic tissues. Active demethylation was reported for the *myosin* gene in differentiating myoblast cells (Lucarelli et al. 2001), Il2 gene upon T cell activation (Bruniquel and Schwartz 2003), the interferon γ gene upon antigen exposure of memory CD8 T cells (Kersh et al. 2006), in the glucocorticoid receptor (GR) gene promoter in adult rat brains upon treatment with the HDAC inhibitor (HDACi) TSA (Weaver et al. 2004), and in neurons as a function of neural activity (Rudenko et al. 2013).

The precise mechanisms governing DNA demethylation are the subject of intense investigation. One proposal has been that a G/T mismatch repair glycosylase also functions as a 5-methylcytosine DNA glycosylase, recognizes methyl cytosines and cleaves the bond between the sugar and the base. The abasic site is then repaired and replaced with a nonmethylated cytosine resulting in demethylation

(Jost 1993). An additional protein with a similar activity was identified as the methylated DNA-binding protein 4 (MBD4; Zhu et al. 2000). Active demethylation early in embryogenesis as well as in somatic cells was also shown to be catalyzed by a nucleotide excision repair mechanism, whereby methylated cytosines are replaced by unmethylated cytosines, which involves the growth arrest and damage response protein Gadd45a and the DNA repair endonuclease XPG (Barreto et al. 2007). It has been proposed that the pathway from methylated to unmethylated DNA involves 5-mC hydroxylation via TET enzymes (Guo et al. 2011). As such, 5-hmC may be an intermediary marker of demethylation. In sum, though a number of biochemical processes were implicated in demethylation, it is unclear how and when these different enzymes participate in shaping and maintaining the overall pattern of methylation and how these activities respond to different environmental exposures.

17.2.7 Targeting DNA Methylation and Demethylation to Specific Genes

A central question regarding gene-specific changes in DNA methylation associated with the environment concerns the targeting of these changes to specific loci in the genome. Because DNA methylating and demethylating enzymes are nonspecific, targeting must be achieved via other mechanisms. There is evidence that chromatin configuration can regulate the accessibility of genes to either DNA methylation or demethylation machineries (Cervoni and Szyf 2001; D'Alessio and Szyf 2006). For example, the HDACi trichostatin A (TSA), which leads to hyperacetylated chromatin, also leads to active DNA demethylation (Cervoni and Szyf 2001). A change in histone acetylation is normally caused by transcription factors that recruit HATs, which may cause histone acetylation and facilitate demethylation. Examples of histone-modifying enzymes shown to interact with DNMT1 are HDAC1, HDAC2, the histone methyltransferases SUV3–9 and enhancer of zeste homolog 2 (EZH2), a member of the multi-protein Polycomb complex PRC2 that methylates H3 histone at the K27 residue (Fuks et al. 2000, 2003; Rountree et al. 2000; Vire et al. 2005). DNMT3a was also shown to interact with EZH2 which targets the DNA methylation-histone modification multi-protein complexes to specific sequences in DNA (Vire et al. 2005).

Sequence-specific transcription factors may target demethylation to specific genes. *Trans*-acting repressors target both histone-modifying enzymes and DNMTs to specific *cis*-acting signals in regulatory regions of particular genes causing gene-specific DNA methylation and chromatin modification. For example, the promyelocytic leukemia PML-RAR fusion protein targets HDAC and DNMTs to its binding sequences and produces de novo DNA methylation of adjacent genes (Di Croce et al. 2002). The intronic kappa chain enhancer and the transcription factor NF-kappaB are required for B-cell-specific demethylation of the kappa immunoglobulin gene (Lichtenstein et al. 1994). As we discuss below, maternal care may be a behavioral mechanism to program gene expression through recruitment of the

transcription factor nerve growth factor-induced protein A (NGFI-A) to a promoter region of the GR in the hippocampus (Weaver et al. 2007).

In summary, we and others have proposed that DNA modifications are maintained in an equilibrium between methylation and demethylation as long as this equilibrium of sequence-specific factors engagement of the genes is maintained (Sasaki et al. 2013; McGowan and Szyf 2010). This process is essential for normal development and the process of tissue-specific cellular differentiation. Physiological or environmental signals, which alter the signaling pathways in the cell, may result in altering this balance by activating or suppressing specific *trans* acting factors.

17.2.8 DNA Methylation and Gene Repression

DNA methylation in critical gene regulatory regions can silence gene expression. There are two main mechanisms by which cytosine methylation suppresses gene expression. The first mechanism involves direct interference of the methylation mark with the binding of a transcription factor to its recognition element in the gene. The interaction of transcription factors with genes is required for activation of the gene; lack of binding of a transcription factor would result in silencing of gene expression (Comb and Goodman 1990; Inamdar et al. 1991). This form of inhibition of transcription by methylation requires that the methylation events occur within the recognition sequence for a transcription factor. A second mechanism is indirect. A certain density of DNA methylation moieties in the region of the gene attracts the binding of methylated-DNA-binding proteins such as MBD1, MBD2, MBD3, and MeCP2, which lead to the formation of a "closed" chromatin configuration and silencing of gene expression (Nan et al. 1997; Hendrich and Bird 1998; Ng et al. 1999; Fujita et al. 1999). For example, MeCP2 recruits other proteins such as SIN3A and histone-modifying enzymes, modifying chromatin conformation via this mechanism (Nan et al. 1997).

17.2.9 Cross Talk Between Chromatin Structure and DNA Methylation

As described previously, DNA methylation can define chromatin structure by recruiting chromatin-modifying enzymes. Emerging evidence indicates that targeting may be a result of both the cross talk between DNA methylation and chromatin modifications, and transcription factor recruitment of DNA-modifying proteins to gene regulatory elements. The loss of DNA methylation will result in the "opening" of chromatin configuration due to increased levels of histone acetylation. Thus, chromatin structure and DNA methylation exhibit a substantial cross talk that creates a feedback loop whereby DNA methylation increases chromatin compaction and transcriptional repression. DNA demethylation likewise increases chromatin activation, further enhancing DNA demethylation (Cedar and Bergman 2009).

17.3 Maternal Care, Epigenetics, and the HPA Axis: Laboratory Animal Studies

Research findings by Weaver, Meaney, Szyf, and colleagues in the early 2000s launched epigenetics to the forefront of research on mechanisms leading from the maternal behavior to long-term programming of gene expression the offspring. Earlier work by the Meaney laboratory and others had established that naturally occurring differences in maternal care in the early postnatal period—during the first week of life in the rat—lead to long-term effects on stress and stress-related behavior. The offspring of mothers who naturally exhibit high levels of care show elevated transcript abundance of the GR in the hippocampus, enhanced negative feedback sensitivity, and a more modest response to stressors in adulthood (Liu et al. 1997). Cross-fostering studies showed that this phenotype is directly attributable to maternal behavior rather than factors related to the prenatal environment, as offspring phenotype was shown to match that of the adoptive mother rather than that of the biological mother (Francis et al. 1999). Weaver and colleagues showed that the accompanying change in GR expression was regulated by DNA methylation of the $GR1_7$ splice variant in the hippocampus by inhibiting the binding of NGFI-A, a transcription factor that drives GR expression (Weaver et al. 2004). GR17 is 1 of at least 11 untranslated first exons of the GR gene. Though it is ubiquitously expressed in virtually all cells, levels of expression of GR vary and are controlled in part by tissue-specific expression of GR exon 1 splice variants (this is also true for the human GR exon 1 as is discussed; Turner and Muller 2005; McCormick et al. 2000). In the hippocampus, GR17 was previously shown to vary in expression as a function of the level of maternal care received (McCormick et al. 2000). Interestingly, relatively high levels of DNA methylation were maintained among the offspring of low maternal care mothers, whereas offspring of high maternal care mothers showed demethylation of this promoter during the first week of life, coinciding with emergence of differences in maternal care between the two litter types. The results implied that DNA demethylation (through a yet unknown process) leads to an increased number of GRs and an attenuated response to stress. DNA methylation differences were stable throughout adulthood in these animals, but were reversible by infusion of trichostatin A (TSA), an HDACi, which also leads to increased gene expression in hundreds of other genes (Weaver et al. 2006). Likewise, lower levels of DNA methylation observed among the offspring of high maternal care mothers resembled that of offspring of high maternal care mothers given central infusions of the methyl donor l-methionine, indicating that enzymes responsible for DNA methylation were poised to act in the adult brain in response to methyl donor.

A recent study has challenged the idea that $GR1_7$ transcript is regulated by DNA methylation of the NGFI-A response element in rats exposed to stress paradigms that lead to altered NGFI-A levels, though stress does appear to modulate the methylation of other CG sites within the $GR1_7$ promoter (Witzmann et al. 2012). It is likely that DNA methylation of $GR1_7$ gene expression involves the binding of additional transcription factors and/or is context and brain region specific. It is also

likely that the $GR1_7$ is itself part of a response mechanism that involves additional splice variants of GR and perhaps other transcription factors.

We performed a microarray analysis of DNA methylation, H3K9 acetylation, and gene expression in a 7-million base pair region containing the GR gene in the rat hippocampus (McGowan et al. 2011). We found that epigenetic differences in adulthood that were associated with early maternal care occurred in clustered regions of up to 100 kbp but were nonetheless exquisitely patterned, whereby increased transcription occurred in conjunction with hyperacetylation and hypermethylation of exons, and hypomethylation of promoters. We found epigenetic differences in association with altered transcription as a function of maternal care across several GR1 splice variants. Large epigenetic differences were noted in proximity to the transcription start site of GR, within the first coding exon (exon 2) and within GR introns, suggesting there may be additional regulation of GR via yet-to-be-identified noncoding RNAs within the GR locus. These data were the first to link epigenetic changes across both coding and noncoding regions in the mammalian brain, and implicate a nonrandom "epigenetic programming" across large-scale loci in response to differences in early care. Accumulating evidence indicates that additional genes in the neural pathway mediating the stress response are epigenetically regulated by DNA methylation of gene regulatory elements in association with early-life stress, for example, arginine vasopressin in the hypothalamus (Murgatroyd et al. 2009), BDNF in the hippocampus (Roth et al. 2009), and GAD67 in the prefrontal cortex (Zhang et al. 2010).

These postnatal programming effects appear to derive from environmentally induced alterations of maternal–neonatal interactions, involving systems that determine methylation patterns of GR gene promoter sequences and additional loci. It will be important to understand the precise nature of the maternal–neonatal interaction that mediates these changes. For example, there is evidence that artificial stimulation of pups with a paintbrush as a substitute for maternal licking can alter DNA methylation of a promoter region so of the estrogen receptor alpha gene in the preoptic area of the hypothalamus (Kurian et al. 2010). These data have important implications for studies of trans-generational impacts related to maternal care via epigenetic mechanisms, through via *behavioral* mechanism of inheritance rather than gametic inheritance, as maternal behavior tends to correlate with the maternal care provided by offspring to their progeny (Champagne et al. 2003).

17.4 Maternal Care, Epigenetics, and the HPA Axis: Human Studies

In the previous section, we discussed evidence from our studies of widespread but specific epigenetic and transcriptional alterations of the GR gene extending far beyond the GR promoter associated with differences in maternal care (McGowan et al. 2011; Murgatroyd et al. 2009; Roth et al. 2009; Zhang et al. 2010). Thus, there is mounting evidence that epigenetic mechanisms coordinate widespread changes in gene expression in response to differences in early maternal care or adversity.

GR promoter DNA methylation has been associated in peripheral blood with a variety of outcomes related to HPA dysfunction. In one of the earliest reports, DNA methylation of GR promoter in infants' cord blood was found to differ as a function of maternal mood during pregnancy and correlate with infants' cortisol response (Oberlander et al. 2008). These data suggest that GR promoter methylation in the brain and in lymphocytes is under epigenetic control as a function of the pre- and postnatal factors. A more recent study indicated that DNA methylation of GR promoter in placenta was associated with birth weight, implicating GR methylation in placental function and suggesting that environmental factors alter metabolic processes in part via epigenetic changes in GR (Filiberto et al. 2011). Other recent research has identified GR DNA methylation as a predictor of treatment outcome in PTSD patients (Yehuda et al. 2013).

We examined postmortem brain tissue from adults with well-characterized life histories to investigate the influence of early-life adversity on GR DNA methylation in adults with a history of trauma. Our focus was on individuals with a history of severe physical or sexual abuse or neglect during childhood, which is common among suicide victims, and is an important risk factor for suicide (Turecki et al. 2012). We examined the GR1F promoter in the hippocampus of human suicide victims and controls (McGowan et al. 2009). Family dysfunction and childhood adversity are linked to altered HPA stress responses and an increased risk for suicide. The promoter region we examined is upstream of one of several untranslated exon 1 splice variants that are known to regulate tissue-specific expression of GR, akin to the function that the GR exon 1 splice variants serve in the rodent (Turner and Muller 2005). The study included three conditions: (1) suicide completers with a history of childhood abuse or severe neglect, (2) suicide completers without a history of childhood abuse or neglect, and (3) individuals who have neither committed suicide nor had a history of childhood abuse or neglect. A fourth group of nonsuicide victims with a history of abuse or neglect was not available, partly due to the fact that tissues from such a "control" group are exceedingly rare, and were unavailable for our study. In this study, we found that the GR gene was differentially methylated among suicide victims with a history of abuse in childhood, but not among suicide victims with a negative history of childhood abuse, compared to control individuals without a history of suicide. The data suggest that epigenetic processes might mediate the effects of the social environment during childhood on hippocampal gene expression and that stable epigenetic marks such as DNA methylation might then persist into adulthood and influence vulnerability for psychopathology through effects on intermediate levels of function such as activity of the HPA axis that regulates the stress response. However, it is still unclear whether the epigenetic aberrations were present in the germ line, whether they were introduced during embryogenesis, or whether they were truly changes occurring during early childhood. We also do not yet know the extent to which parental factors per se play a role in this phenotype. Despite these important caveats, these data were the first to link the early-life environment to changes in the GR gene in humans. The data parallel that in the rodent study mentioned above, though in a very different context.

We have applied high-throughput approaches to examining DNA methylation, chromatin modifications, and mRNA expression in gene regulatory, coding, intragenic, and intergenic regions in humans in a study that paralleled that described above in rats. We analyzed the GR gene locus by interrogating a 7-Mbp region containing the GR gene in hippocampi of adult suicide victims who were abused early in life compared to controls using high-throughput DNA microarray (Suderman et al. 2012). The GR gene locus shows substantial conservation with the same locus in rodents, with an almost identical order or orthologous genes across the locus. Like the study in the rat (McGowan et al. 2011), methylation levels were nonrandomly distributed across the locus, indicating that stochastic processes are unlikely to account for the range of variation that we observed in this study. Proximal to the GR gene itself, we found a large region hypermethylated in suicide completers relative to controls within the first coding exon of the GR gene and its proximal promoters, extending previous observations of hypermethylation of the GR1F promoter among suicide victims with a history of abuse (McGowan et al. 2009). This analysis also revealed differences in DNA methylation in intragenic regions of the GR gene. At this time, we can only speculate that unrecognized noncoding RNAs may reside within this region and affect GR expression. Other differences were discovered within coding regions and the 3′ UTR of the GR gene. These data suggest that GR is epigenetically labile in response to the early-life social environment in both rodents and humans, though the specific alterations that we observed are not identical in both species (Suderman et al. 2012). Nevertheless, the data indicate that the animal model of parental care may have broad applicability for translational studies aimed at understanding the consequences of epigenetic modification of GR in humans.

Though the most cost-effective means of targeting specific loci for epigenetic analyses remain microarray approaches combined with immunoprecipitation, such studies are not without limitations. Such approaches suffer from a lack of resolution (~200 bp) compared to single-nucleotide resolution sequencing-based analyses. As the cost of sequencing continues to decrease, it is now becoming feasible to employ sequencing-based epigenetic analyses of DNA methylation (via meDIP-Seq or Bisulfite Sequencing [BIS-seq]) and chromatin modifications (via ChIP-seq). These two have important limitations, as have been reviewed elsewhere (e.g., Bock 2012). High-throughput studies such as the ones described above in the rat and human open up a number of questions—undoubtedly more than are answered. It is clear that these technological advances that allow whole-epigenome analysis must be coupled with equally powerful phenotypic screens using appropriate cell types and conditions.

17.5 Conclusions and Prospective

A more complete understanding of the role of epigenetic mechanisms in perinatal programming will be afforded by studies that address several basic questions. First, in what contexts is the epigenome labile in response to early environment? Are

there indeed critical time windows for the influence of the environment on epigenetic trajectories? A number of studies have linked early-life events to changes in neuroplasticity that have a lasting impact of endocrine systems mediating the response to stress (McEwen 2012). It is not always clear, however, which cell types are relevant to the question under study. This is particularly problematic for studies in humans, where access to neural tissue is nonexistent or limited. Peripheral cells such as peripheral blood mononuclear cells (PBMCs) offer an avenue to examine the HPA, as PBMCs are sensitive to endocrine modulation of HPA [ref]. Whole blood has also been used, but each tissue type is known to be sensitive to differences in constituent cell numbers, which may bias the results (Lam et al. 2013; Suderman et al. 2013). However, in studying environmental impacts prospectively in children, it is not often possible to obtain blood samples and other tissues must be used. The most commonly used tissues in such epigenetic studies are buccal cells from mouth swabs or saliva. Intriguingly, there is some evidence that such tissue is responsive to early-life adversity, though perhaps not via epigenetic changes in GR per se (Essex et al. 2013). Buccal cells complement studies of adversity in neurons in the sense that they do represent cells with a common embryonic origin. Such studies will provide a valuable means of resampling to examine epigenetic variance over time and with interventions. In animal studies, a goal going forward for translational work will be to identify labile epigenetic regions like the GR that can be assessed in brain and blood in order to generate hypotheses and biomarkers that can be examined in humans. Such research stands to offer critical insights into the manner by which the biological embedding occurs during the perinatal period.

Conflict of Interest The authors declare no conflicts of interest.

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