Epigenetics and Human Health

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Epigenetics and Neuroendocrinology

Clinical Focus on Psychiatry

Volume 1

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Foreword

The field of neuroendocrinology has evolved from initial studies on the hypothalamic control of pituitary secretion to the study of multilayered reciprocal interactions between the central nervous system (CNS) and the endocrine system. Together, they serve to coordinate a vast range of physiological responses in order to maintain homeostasis. At the same time, neuroendocrine systems can undergo dynamic, potentially lasting, adjustments in preset thresholds and regulatory set points during critical periods of development and beyond. Such adjustments, commonly thought of as adaptation, can enhance the capacity of an organism to cope with recurrent challenges but may also increase the risk for certain diseases. Importantly, molecular epigenetic mechanisms are increasingly recognized for their role both in the development and maturation of the neuroendocrine system as well as for their role as a molecular interface in the mediation of multifaceted gene-environment interactions.

Epigenetics and Neuroendocrinology – Focus on Psychiatry – addresses current advances in the understanding of molecular epigenetic mechanisms for the function and adjustment of neuroendocrine systems and their impact on trauma- and stress-related psychiatry disorders.

With the beginning of the last century, experimental hypophysectomy (Crowe et al. 1910) and stereotactic hypothalamic lesions (Hetherington and Ranson 1940) demonstrated a close interaction between the hypothalamus and the pituitary. These and following studies clearly established that an intact hypothalamus is necessary for normal endocrine function although the mechanisms mediating these effects remained obscure.

This gap was filled when several groups, namely, the one of Ernst and Berta Scharrer (Scharrer 1987), discovered that neurons in the hypothalamus are the origin of the axons that constitute the neural, posterior lobe. Further refined anatomical studies (Wislocki and King 1936) revealed the role of pituitary portal vessels in linking the median eminence of the hypothalamus and the anterior pituitary and grounded today's hypophyseal-portal-chemotransmitter hypothesis.

The decades to follow witnessed major progress in the identification of the factors that mediate the communication between the hypothalamus and the pituitary

and were crowned by the isolation of several putative peptide hormone releasing factors by Andrew Schally and Roger Guillemin (Guillemin 1978; Schally 1978). These neuropeptides turned out to be the long-sought functional link between the CNS and the endocrine systems in the control of reproduction, growth, metabolism, and the stress response. Epigenetic regulation of these releasing factor genes during critical time windows of development and their relevance for the onset, progression, and course of major depression and trauma presents an important aspect of this book (Part I).

Following on, the cloning and characterization of specific G protein-coupled receptors bound by hypothalamic releasing factors enabled elucidation of the underlying signaling pathways and opened up the prospect of tailored treatments (Griebel and Holsboer 2012). Experience-dependent epigenetic (de)regulation of the encoding receptor alleles has emerged as an important step in the pathology of several psychiatric disorders (Part III).

Although the discipline of neuroendocrinology has focused traditionally on the clinical function of hypothalamic releasing factors in reproduction and development, metabolism, fluid balance, and stress, the field has expanded over the last decades to embrace the multilayered interactions of the endocrine and nervous system in the control of homeostasis. Within this framework the concept of endocrine psychiatry emerged at the beginning of the twentieth century as a new discipline with Manfred Bleuler as one of its leading protagonists (Bleuler 1965). This development also gave birth to the field of psychoneuroendocrinology comprising the clinical study of hormone fluctuations and their relationship to human behavior. Certain mood disorders were shown to be associated with neuroendocrine or hormonal changes affecting brain function while otherwise certain endocrine disorders were shown to associate with psychiatric diseases. New areas in these research fields include, among others, neurosecretion, neurotransmission, receptor pharmacology, transcriptional regulation, and most recently molecular epigenetics (Allis et al. 2015).

Homeostatic systems integrate endocrine, autonomic, and behavioral outcomes by connecting classical neuroendocrine axes to neuronal inputs and refined feedback loops to maintain a dynamic equilibrium (Part I). In the classical stress concept this well-balanced state is challenged by certain physical and psychological events termed "stressors" (Fink 2007). These stressors also trigger neuroendocrine and behavioral responses with the aim to reinstate homeostasis. Excessive, inadequate, or enduring stress responses can trigger epigenetic mechanisms that inscribe long-lasting memory traces into the methylome of exposed individuals and may act in conjunction with certain genetic predispositions as risk factors for various psychiatric diseases (Parts I, III–V).

Early life comprises a period of both great vulnerability and great opportunity for brain development (Shonkoff and Phillips 2000). A growth-promoting environment filled with attentive social interactions prepares the highly plastic developing brain to evolve optimally. Conversely, adverse early life experiences can result in faulty brain circuitry and leave lasting, if not lifelong, molecular epigenetic footprints at the hypothalamic-pituitary-adrenal axis (Parts I, III–V). Persuasive evidence has

been gained for a role of experience-dependent molecular epigenetic marks in the mediation between early life adversity and later psychopathology (Heim and Binder 2012; Hoffmann and Spengler 2012).

Our sex plays a fundamental role in our daily lives and the timing of onset, prevalence, clinical course, and treatment response for various mental disorders (Part II). Sexual differentiation of the brain occurs during a perinatal-sensitive time window as a result of gonadal hormone-driven activational and organizational effects on neuronal templates. Molecular epigenetic mechanisms contribute to these processes and are themselves under the control of sex hormones (Part II). Epigenetic programming of neuroendocrine and behavioral phenotypes is sex dependent (Parts I–V), indicative of a tight interplay between sex differences in molecular brain epigenetics and gonadal hormones. In support of this view, loss of transcriptional repression is a key mechanism underlying the onset of puberty in females and is triggered by molecular epigenetic cues (Part II).

Integrated analysis of neuroendocrine systems can advance our insight into the relationship from epigenetically mediated adaptation to disease (Choi 2010). In positive feedback systems, the controlled variable increases hormone output but decreases it in case of negative feedback systems. The hypothalamic-pituitary-adrenal axis presents a classical example where glucocorticoid receptors, encoding ligand-gated transcription factors (Bunce and Campbell 2010), sense the concentration of steroid hormones and terminate the output of the system (Part I). Receptors of classical steroids, the glucocorticoid, mineralocorticoid, progesterone, androgen, and estrogen receptors reside in the cytoplasm in a complex with chaperon proteins. Following ligand binding, they translocate to the nucleus to bind predominantly as homodimers at well-defined response elements. Subsequently, they confer transcriptional regulation by the combinatorial recruitment of multiple cofactor complexes containing various enzymatic activities catalyzing site-specific histone modifications underlying an "open" (transcriptionally active) or "closed" (transcriptionally inactive) chromatin structure (Part I). The expression of nuclear receptors can be epigenetically programmed by early-life experiences in a tissue-specific manner and represents an important risk factor for the development of various psychiatry disorders (Part V). Similarly, allele-specific epigenetic marking of FKBP51, encoding a key chaperon for glucocorticoid receptor function, has been discovered as a molecular mechanism underlying gene-environment interactions in stress-related psychiatric disorders (Part III). Moreover, the DNA methylation status at glucocorticoid receptor response elements can regulate cell-type specific enhancer activity (Part I). Together, these findings exemplify how molecular epigenetic mechanisms can operate at multiple levels to control negative feedback regulation of the hypothalamic-pituitary-adrenal axis.

For the past 60 years, the genome has been viewed as an immutable master plan that has been laid down with the inception of our lives with DNA as the heritable molecule that carries information about phenotypes from parent to offspring (Jablonka et al. 2005). Experimental studies in different animal models and observational findings in humans suggest, however, that stressful exposures during pregnancy, birth, or adolescence can be passed down to the offspring (intergenerational)

and subsequent generations (transgenerational) to affect sex-dependently neuroendocrine and behavioral responses (Part IV). Some routes require the continuous presence of the initial trigger and result from behavioral and social transfer while others may uncouple from the initial trigger and rely solely on the molecular transfer through the germ cells. Molecular epigenetic mechanisms seem to underpin these effects, and diffusible factors, in particular hormonal signals and possibly RNAs, might explain epigenetic inheritance via the gametes. The impetus of such transmissible effects for the chemistry of our children's DNA and for evolutionary medicine awaits still careful investigations (Gluckman et al. 2010).

A concluding open question is how to alleviate epigenetically encoded disease risks in psychiatric disorders. Epigenetic biomarkers offer a promising tool to identify individuals at risk and for outpatient monitoring (Part V). If social experiences and adverse stressors are important determinants, this may be good news; physiological stress responses that depend strongly on epigenetic programming may be more amenable to psychotherapeutic interventions than hardwired genetic factors. Therapeutic approaches that aim to revise perceptions psychologically may complement personalized pharmacological treatments, and possibly there are interactive psychological and pharmacological interventions that work better than either type of approach alone.

July 2015 Dietmar Spengler
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Glossary

Acetylation The introduction, via an enzymatic reaction, of an acetyl group to an organic compound, for instance to *histones* or other proteins.

Agouti gene The agouti gene (A) controls fur colour through the deposition of yellow pigment in developing hairs. Several variants of the gene exist, and for one of these (Agouti Variable Yellow, A^{vy}) the expression levels can be heritably modified by *DNA methylation*.

Alleles Different variants or copies of a gene. For most genes on the chromosomes, there are two copies: one copy inherited from the mother, the other from the father. The DNA sequence of each of these copies may be different because of genetic polymorphisms.

Assisted reproduction technologies (ART) The combination of approaches that are being applied in the fertility clinic, including *IVF* and *ICSI*.

5-Azacytidine A cytidine analog in which the 5 carbon of the cytosine ring has been replaced with nitrogen. 5-azacytidine is a potent inhibitor of mammalian *DNA methyltransferases*.

Bisulfite genomic sequencing A procedure in which bisulfite is used to deaminate cytosine to uracil in genomic DNA. Conditions are chosen so that 5-methylcytosine is not changed. PCR amplification and subsequent DNA sequencing reveals the exact position of cytosines which are methylated in genomic DNA.

Bivalent chromatin A chromatin region that is modified by a combination of histone modifications such that it represses gene transcription, but at the same time retains the potential of acquiring gene expression,.

Brno nomenclature Regulation of the nomenclature of specific histone modifications formulated at the Brno meeting of the NoE in 2004. Rules are: <Histone > <amino-acid position > <modification type > <type of modification>. Example: H3K4me3 = trimethylated lysine-4 on histone H3

Bromo domain Protein motif found in a variety of nuclear proteins including transcription factors and HATs involved in transcriptional activation. Bromo domains bind to histone-tails carrying acetylated lysine residues.

Cell fate The programmed path of differentiation of a cell. Although all cells have the same DNA, their cell fate can be different. For instance, some cells develop into brain, whereas others are the precursors of blood. Cell fate is determined in part by the organisation of *chromatin* – DNA and the histone proteins – in the nucleus.

Cellular memory (epigenetic) Specific active and repressive organisations of chromatin can be maintained from one cell to its daughter cells. This is called *epigenetic inheritance* and ensures that specific states of gene expression are inherited over many cell generations.

ChIP See *chromatin immuno-precipitation*.

ChIP on chip After chromatin immunoprecipitation, DNA is purified from the immunoprecipitated chromatin fraction and used to hybridise arrays of short DNA fragments representing specific regions of the genome.

ChIP Seq Sequencing of the totality of DNA fragments obtained by ChIP to determine their position on the genome. Sequencing is usually preceeded by PCR amplification of ChIP derived DNA to increase its amount.

Chromatid In each somatic cell generation, the genomic DNA is replicated in order to make two copies of each individual chromosome. During M phase of the cell cycle, these copies – called chromatids – are microscopically visible one next to the other, before they get distributed to the daughter cells.

Chromatin The nucleo-protein-complex constituting the chromosomes in eukaryotic cells. Structural organisation of chromatin is complex and involves different levels of compaction. The lowest level of compaction is represented by an extended array of *nucleosomes*.

Chromatin immuno-precipitation (ChIP) Incubation of chromatin fragments comprising one to several nucleosomes, with an antiserum directed against particular (histone) proteins or covalent modifications on proteins. After ChIP, the genomic DNA is purified from the chromatin fragments brought down by the antiserum and analysed.

Chromatin remodelling Locally, the organisation and compaction of chromatin can be altered by different enzymatic machineries. This is called chromatin remodelling. Several chromatin remodelling proteins move *nucleosomes* along the DNA and require ATP for their action.

Chromo domain (chromatin organization modifier domain) Protein-protein interaction motif first identified in *Drosophila melanogaster HP1* and *polycomb group proteins*. Also found in other nuclear proteins involved in transcriptional silencing and heterochromatin formation. Chromo domains consist of approx. 50 amino acids and bind to histone tails that are methylated at certain lysine residues.

Chromosomal domain In higher eukaryotes, it is often observed that in a specific cell type, chromatin is organised (eg, by *histone methylation*) the same way across hundreds to thousands of kilobases of DNA. These 'chromosomal domains' can comprise multiple genes that are similarly expressed. Some chromosomal domains are controlled by *genomic imprinting*.

CpG dinucleotide A cytosine followed by a guanine in the sequence of bases of the DNA. *Cytosine methylation* in mammals occurs at CpG dinucleotides.

CpG island A small stretch of DNA, of several hundred up to several kilobases in size, that is particularly rich in *CpG dinucleotides* and is also relatively enriched in cytosines and guanines. Most CpG islands comprise promoter sequences that drive the expression of genes.

Cytosine methylation In mammals, DNA methylation occurs at cytosines that are part of *CpG dinucleotides*. As a consequence of the palindromic nature of the CpG sequence, methylation is symmetrical, i.e., affects both strands of DNA at a methylated target site. When present at promoters, it is usually associated with transcriptional repression.

Deacetylation The removal of acetyl groups from proteins. Deacetylation of histones is often associated with gene repression and is mediated by histone deacetylases (HDACs).

"de novo" DNA-methylation The addition of methyl groups to a stretch of DNA which is not yet methylated (acquisition of 'new' DNA methylation).

Disomy The occurrence in the cell of two copies of a chromosome, or part of a chromosome, that are identical and of the same parental origin (uniparental disomy).

DNA-demethylation Removal of methyl groups from DNA. This can occur 'actively', i.e. by an enzymatically mediated process, or 'passively', when methylation is not maintained after DNA replication.

DNA-methylation A biochemical modification of DNA resulting from addition of a methyl group to either adenine or cytosine bases. In mammals, methylation is essentially confined to cytosines that are in *CpG dinucleotides*. Methyl groups can be removed from DNA by DNA-demethylation.

DNA methyltransferase Enzyme which puts new (***de novo***) methylation onto the DNA, or which maintains existing patterns of DNA methylation.

Dosage compensation The X chromosome is present in two copies in the one sex, and in one copy in the other. Dosage compensation ensures that in spite of the copy number difference, X-linked genes are expressed at the same level in males and females. In mammals, dosage compensation occurs by inactivation of one of the X chromosomes in females.

Early-life adversity (ELA) Parental maladjustment (mental illness (frequently MDD), substance abuse, violence, and criminality), maltreatment (sexual abuse, physical abuse, or neglect), interpersonal loss (parental death or divorce, separation from parents or caregivers), life threatening childhood physical illness in the respondent, or severe childhood family financial distress are leading sources of ELA and typically associate with early-life stress (ELS).

Most studies have focused on childhood trauma, in particular sexual and physical abuse though neglect during early childhood is the most common form of maltreatment, accounting for more than three quarters of all maltreatment cases. ELA is a strong risk factor for the development of various psychiatric diseases, particularly major depressive disorder.

Embryonic stem (ES) cells Cultured cells obtained from the inner cell mass of the blastocyst, and for human ES cells, possibly also from the epiblast. These cells are totipotent; they can be differentiated into all different somatic cell lineages.

ES-like cells can be obtained by dedifferentiation *in vitro* of somatic cells (see *iPS cells*).

Endocrine disruptor A chemical component which can have an antagonistic effect on the action of a hormone (such as on estrogen) to which it resembles structurally. Some pesticides act as endocrine disruptors and have been found in animal studies to have adverse effects on development, and for some, to induce altered *DNA methylation* at specific loci. A well characterised endocxrine disruptor is *Bisphenol-A*, a chemical used for the productions of certain plastics.

Enhancer A small, specialised sequence of DNA which, when recognised by specific regulatory proteins, can enhance the activity of the promoter of a gene(s) located in close vicinity.

Epi-alleles Copies of a DNA sequence or a gene which differ in their epigenetic and/or expression states without the occurrence of a genetic mutation.

Epigenesis The development of an organism from fertilisation through a sequence of steps leading to a gradual increase in complexity through differentiation of cells and formation of organs.

Epigenetics The study of heritable changes in gene function that arise without an apparent change in the genomic DNA sequence. Epigenetic mechanisms are involved in the formation and maintenance of cell lineages during development, and, in mammals, in *X-inactivation* and *genomic imprinting*, and are frequently perturbed in diseases.

Epigenetic code Patterns of DNA methylation and histone modifications can modify the way genes on the chromosomes are expressed. This has led to the idea that combinations of epigenetic modifications can constitute a code on top of the genetic code which modulates gene expression.

Epigenetic inheritance The somatic inhertance, or inheritance through the germ line, of epigenetic information (changes that affect gene function, without the occurrence of an alteration in the DNA sequence).

Epigenetic marks Regional modifications of DNA and chromatin proteins, including *DNA methylation* and histone methylation, that can be maintained from one cell generation to the next and which may affect the way genes are expressed.

Epigenetic reprogramming The resetting of *epigenetic marks* on the genome so that these become like those of another cell type, or of another developmental stage. Epigenetic reprogramming occurs for instance in *primordial germ cells*, to bring them back in a 'ground state'. Epigenetic reprogramming and dedifferentiation also occur after *somatic cell nuclear transfer*.

Epigenome The epigenome is the overall epigenetic state of a particular cell. In the developing embryo, each cell type has a different epigenome. Epigenome maps represent the presence of DNA methylation, histone modification and other chromatin modifications along the chromosomes.

Epigenotype The totality of epigenetic marks that are found along the DNA sequence of the genome in a particular cell lineage or at a particular developmental stage.

Epimutation A change in the normal epigenetic marking of a gene or a regulatory DNA sequence (e.g., a change in DNA methylation) which affects gene expression.

Euchromatin A type of chromatin which is lightly staining when observed through the microscope at interphase. Euchromatic *chromosomal domains* are loosely compacted and relatively rich in genes. The opposite type of chromatin organisation is *heterochromatin*.

Genomic imprinting An epigenetic phenomenon which affects a small subset of genes in the genome and results in mono-allelic gene expression in a parent-of-origin dependent way (for a given pair of alleles uniformly either the maternally or paternally derived copy is active).

Germ line specific stem cells Cells derived from undifferentiated germ cells which can be maintained without alterations in their characteristics through many cell divisions.

Glucocorticoid (GC) Main stress hormones released from the adrenal glands following activation of the hypothalamic-pituitary-adrenal axis. Typical glucocorticoids are corticosterone in animals, and cortisol in humans. Sustained up-regulation of GCs is found following exposure to early-life adversity and during the course of major depressive disorders.

Heterochromatin A type of chromatin which is darkly staining when observed through the microscope at interphase., Heterochromatic chromosomal domains, found in all cell types, are highly compacted, rich in repeat sequences, and show little or no gene expression. Extended regions of heterochromatin are found close to centromeres and at telomeres.

Histone acetylation Post-translational modification of the ε-amino group of lysine residues in histones catalyzed by a family of enzymes called *histone acetyltransferases (HATs)*. Acetylation contributes to the formation of decondensed, transcriptionally permissive chromatin structures and facilitates interaction with proteins containing *bromo domains*.

Histone acetyltransferase (HAT) An enzyme that acetylates (specific) lysine amino acids on histone proteins.

Histone code Theory that distinct chromatin states of condensation and function are marked by specific histone modifications or specific combinatorial codes (see also epigenetic code).

Histone deacetylase (HDAC) An enzyme that removes acetyl groups from histone proteins. This increases the positive charge of histones and enhances their attraction to the negatively charged phosphate groups in DNA.

Histone-demethylase (HDM) Proteins catalysing the active enzymatic removal of methyl groups from either lysine or arginine residues of histones. Prominent examples are LSD1 and Jumonji proteins.

Histone methylation Post-translational methylation of amino acid residues in histones catalysed by *histone methyltransferases (HMTs).* Histone methylation is found at arginine as mono- or di-methylation and lysine as mono-, di- or trimethylation. Modifications are described depending on the position and type

of methylation (mono, di, tri-methylation) according to the *Brno nomenclature*. Different types of methylation can be found in either open trancriptionally active or silent (repressive) chromatin (*histone code*). Methylated lysine residues are recognized by proteins containing *chromo domains*.

Histone methyltransferase (HMT) Enzymes catalysing the transfer of methyl groups from S-adenosyl-methionine (SAM) to lysine or arginine residues in histones.

Hypothalamic-pituitary-adrenal axis (HPA axis) Activated in response to stress, neurons in the hypothalamus release two neuropeptides called corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Their release triggers the subsequent secretion and release of another factor called adrenocorticotropin (ACTH) from the pituitary gland, situated at the basis of the brain. When ACTH is secreted by the pituitary gland, it travels in the blood and reaches the adrenal glands, which are located above the kidneys, and triggers secretion of the so-called stress hormones. There are two main stress hormones, the glucocorticoids, and the catecholamines (epinephrine and norepinephrine).

Imprinted genes Genes that show a parent-of-origin specific gene expression pattern controlled by epigenetic marks that originate from the germ line.

Imprinted X-inactivation Preferential inactivation of the paternal X-chromosome in rodents (presumably also humans) during early embryogenesis and in the placenta of mammals

Imprinting See *genomic imprinting*

Imprinting control region (ICR) Region that shows germ line derived parent of origin dependent epigenetic marking which controls the imprinted expression of neighbouring imprinted genes

Induced pluripotent stem cells (iPS) Cells derived from differentiated somatic cells by in vitro reprogramming. Reprogramming is triggered by the activation of pluripotency factor genes and cultivation in ES-cell medium. iPS cells are capable to generate all cell types of an embryo.

Inner cell mass (ICM) Cells of the inner part of the blastocyst forming the embryo proper. Inner cell mass cells are the source for ES cells.

Intracytoplasmic sperm injection (ICSI) Capillary mediated injection of a single sperm into the cytoplasm of an oocyte followed by activation to promote directed fertilization.

In vitro fertilisation (IVF) Fertilisation of a surgically retrieved oocyte in the laboratory, followed by a short period of in vitro cultivation before the embryo is transferred back into the uterus to allow development to term.

Isoschizomers Restriction enzymes from different bacteria which recognize the same target sequence in DNA. Often these enzymes respond differently to methylation of bases within their target sequence, which may make them important tools in DNA-methylation analysis. Thus, *Msp*I cuts both CCGG and C5mCGG, whereas *Hpa*II cuts only the unmethylated sequence.

Locus control region (LCR) Region marked by insulator functions and DNase hypersensitive sites. LCRs contain binding sites for insulator proteins and enhancer binding proteins. LCRs control the domain specific developmentally regulated expression of genes by long range interactions with gene promoters.

Major depressive disorder (MDD) A world-wide leading mood disorder (also known as major depression (MD), unipolar depression, or as recurrent depression in case of repeated episodes) characterized by a pervasive and persistent low mood that is accompanied by low self-esteem and by a loss of interest or pleasure in normally enjoyable activities. MDD is a disabling condition that adversely affects a person's family, work or school life, sleeping and eating habits, and general health.

Maternal effects Long-term effects on the development of the embryo triggered by factors in the cytoplasm of the oocyte.

Methyl-binding domain (MBD) Protein domain in Methyl-CpG-binding proteins (MBPs) responsible for recognizing and binding to methylated cytosine residues in DNA. Proteins containing MBDs form a specific family of proteins with various molecular functions.

Methyl-CpG-binding proteins (MBPs) Proteins containing domains (such as MBD) binding to 5-methyl-cytosine in the context of CpG dinucleotides. MBPs mostly act as mediators for molecular functions such as transcriptional control or DNA repair.

Non-coding RNA (ncRNA) RNA transcripts that doe not code for a protein. ncRNA generation frequently involves RNA processing.

Non-Mendelian inheritance Inheritance of genetic traits that do not follow Mendelian rules and/or cannot be explained in simple mathematically modelled traits.

Nuclear periphery Region around the nuclear membrane characterized by contacts of the chromosomes with the nuclear lamina.

Nuclear (chromosomal) territory Cell type specific areas within the nucleus occupied by specific chromosomes during interphase (G1).

Nucleolus Specific compartments within the nucleus formed by rDNA repeat domains. Nucleoli are marked by specific heterochromatic structures and active gene expression.

Nucleosome Fundamental organisational unit of chromatin consisting of 147 base pairs of DNA wound around a histone octamer.

Pluripotency Capacity of stem cells to form all cell types of an embryo including germ cells.

Polycomb group proteins Epigenetic regulator proteins forming multiprotein complexes (PRCs = polycomb repressive complexes). Polycomb group proteins posess enzymatic properties to control the maintenance of a suppressed state of developmentally regulated genes, mainly through histone methylation and ubiquitination.

Position effect variegation (PEV) Cell/tissue specific variability of gene expression controlled by the temporal inheritance of certain epigenetic states. PEV is a consequence of variable formation of heterochromatin across the respective gene. A classical example of PEV is found in the certain mutations leading to variegated eye pigmentation in *Drosophila* eyes.

Posttraumatic stress disorder (PTSD) This condition may develop after a vulnerable person is exposed to one or more traumatic events, such as major stress, sexual assault, terrorism, or other threats on a person's life. Characteristic symptoms

comprise disturbing recurring flashbacks (re-experiencing symptoms), avoidance or numbing of memories of the event, and hyperarousal, which continue for more than a month after the occurrence of a traumatic event.

Primordial germ cell Mammalian cells set aside during early embryogenesis which migrate through the hind gut of the developing mammalian embryo into the "Gonadenanlagen" to form founder cells of the latter germ line.

Protamines Small, arginine-rich proteins that replace histones late in the haploid phase of *spermatogenesis* (during *spermiogenesis*). They are thought to be essential for sperm head condensation and DNA stabilization. After fertilization protamines are removed from paternal chromosomes in the mammalian zygote.

RNA interference (RNAi) Posttranscriptional regulatory effects on mRNAs (control of translation or stability) triggered by processed ds and ss small RNA (si-, mi-, pi RNAs) molecules. Effects are propagatesd by enzymatic complexes such as RISC containing the small RNAs bound by Argonaute proteins.

SAHA Suberoylanilide hydroxamic acid, an inhibitor of ertain histone deactylases, leading to enhanced levels of histone acetylation. See also *TSA*.

S-adenosylhomocysteine (SAH) Hydrolysed product formed after the methylation reaction catalyzed by DNA- and *histone methyltransferases* using SAM as methyl group donor. SAH is a competitive inhibitor of SAM for most methyltransferases.

S-adenosyl methionine (SAM) A cofactor for all DNA- (DNMTs) and histone-methyltransferases (HMTs) providing the methyl group added to either cytosines (DNA) or histones (arginine or lysine).

SET domain A domain found in virtually all lysine-specific *histone methyltransferases (HMTs)*. A protein-protein interaction domain required for HMT activity and modulation of chromatin structure, frequently associated with cysteine-rich Pre-SET and Post-SET domains.

Silencer Element in the DNA to which proteins bind that inhibit transcription of a nearby promoter. Silencer elements are recognized and bound by silencer proteins.

siRNAs Small interfering RNAs, RNAs in the size range of 21–24 nucleotides derived from double stranded long RNAs cleaved by Dicer. siRNAs are incorporated into the RISC complex to be targeted to complementary RNAs to promote cleavage of these mRNAs.

Somatic cell nuclear transfer (SCNT) Transfer of the nucleus of a somatic cell into an enucleated oocyte using a glas capillary to form an SCNT-zygote. After activation of the zygote the genome of the nucleus derived from the somatic cells become reprogrammed to start development.

Spermatogenesis The process by which spermatogonia develop into mature spermatozoa. Spermatozoa (sperm) are the mature male gametes. Thus, spermatogenesis is the male version of gametogenesis.

Spermiogenesis The final stage of spermatogenesis which sees the maturation of spermatids into mature, motile spermatozoa (sperm). During this stage, cells no longer divide and undergo a major morphological transformation. In addition, at most of the genome, histone proteins are replaced by the more basic *protamines*.

Stem cell Non-committed cell which has the capacity to self renew and divide many times giving rise to daughter cells which maintain the stem cell function. Stem cells have the property to differentiate into specialized cells.

Stress A stressor is any event that can activate a physiological stress response, e.g., the body's reaction to the event. Consequently, stress is an inferred internal state, based on the physiological stress response. When a situation is interpreted as being stressful, e.g., novel and possibly threatening (inferred state), this triggers a stress response that is typically represented by the activation of the HPA-axis (see hypothalamic-pituitary-adrenal axis).

Totipotency Capacity of stem cells to produce all cell types required to form a mammalian embryo, i.e. embryonic and extraembryonic cells ***(see Pluripotency)***. Totipotent cells are formed during the first cleavages of the embryo.

Trithorax group proteins Proteins containing a thritorax like bromo-domain: They are usually involved in recognizing histone modifications marking transcriptionally active regions and contribute to maintenance of activity.

Trophoblast Cells of the blastoderm forming the placental tissues in mammals.

TSA ***Trichostatin-A***, an inhibitor of certain types of histone-deacetylases.

X chromosome inactivation Epigenetically controlled form of *dosage compensation* in female mammals resulting in transcriptional silencing of genes on surplus X-chromosomes. X-chromosome inactivation is triggered by the non-coding RNA Xist and manifested by various epigenetic modifications including histone methylation, histone deacetylation and DNA-methylation.

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From Vulnerability to Neurotoxicity: A Developmental Approach to the Effects of Stress on the Brain and Behavior

S.J. Lupien, I. Ouellet-Morin, C.M. Herba, R. Juster, and B.S. McEwen

Abstract Many studies published over the last two decades show that chronic exposure to stress hormones, from the prenatal to aging period, can have deleterious effects on brain structures involved in cognition and mental health. In this selective review of the literature, we show that specific effects on the brain, behavior, and cognition emerge as a function of the timing and the duration of exposure to stress. We present the effects of stress on the brain and behavior for different periods, e.g., prenatal and postnatal stress, stress in adolescence and adulthood, and the effects of stress on the aging brain. We first summarize the literature on the effects of stress on the brain and behavior across the lifespan in animal models, and thereafter, we present a translational view of the effects of stress on the brain and behavior in

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humans. In order to better understand these effects, we summarize models that have been recently proposed to explain why different disorders emerge in populations exposed to stress at different moments of their lives. In conclusion, we discuss the importance of developing early interventions to prevent the negative effects of stress on brain development.

Keywords Stress • Glucocorticoids • Animals • Humans • Lifespan • Brain

1.1 Introduction

Contrary to popular belief, children and adolescents are just as capable as adults of experiencing stress and the stress-related health outcomes that ensue (Lohman and Jarvis 2000). The effects of stress on the well-being of children and adolescents are substantial, as stress has been shown to increase incidence of psychiatric problems at this period of development (Goodyer et al. 1996; Rudolph and Hammen 1999; Hudziak et al. 2000; Angold et al. 2002). Adolescence is also a period in which the long-lasting effects of earlier stress become evident. As we age, stress hormones have important effects on the brain of adults and older adults. Younger and older adults are also sensitive to acute effects of stress on memory, and older adults are particularly vulnerable to the effects of chronic stress on the brain. In this first section, we review the effects of stress on the brain, behavior, and cognition from the prenatal period to old age in both animals and humans. In the second section of the chapter, we present various models that delineate the effects of stress on the brain and the corresponding disorders/deficits that can result from exposure to stress at different moment of brain development. In the last section, we discuss epigenetic effects of stress on the brain and how intervening early in life could lead to new windows of opportunity to prevent the deleterious effects of stress on brain development.

1.2 What Is Stress?

1.2.1 The Stress Response

A stressor is any event that can activate a physiological stress response, e.g., the body's reaction to the event (Selye 1975, 1998). Consequently, stress is an inferred internal state, based on the physiological stress response. When a situation is interpreted as being stressful, e.g., novel and possibly threatening (inferred state), this triggers a stress response that is represented by the activation of the hypothalamic–pituitary–adrenal (HPA) axis whereby neurons in the hypothalamus release a hormone called corticotropin-releasing hormone (CRH). The release of CRH triggers the subsequent secretion and release of another hormone called adrenocorticotropin

(ACTH) from the pituitary gland, also located in the brain. When ACTH is secreted by the pituitary gland, it travels in the blood and reaches the adrenal glands, which are located above the kidneys, and triggers secretion of the so-called stress hormones. There are two main stress hormones, the glucocorticoids (called corticosterone in animals and cortisol in humans) and the catecholamines (epinephrine and norepinephrine; see Fig. 1.1).

In humans, cortisol secretion shows pronounced circadian rhythmicity, where concentrations are at their highest in the morning (the circadian peak), progressively decline from late afternoon to early nocturnal periods (the circadian trough), and show abrupt elevations after the first few hours of sleep. The diurnal elevation of cortisol can be thought of as a wake-up signal to increase activity and hunger at the beginning of the active period in both nocturnally and diurnally active animal species (McEwen et al. 1993). The acute secretion of glucocorticoids and catecholamines in response to a stressor constitutes the primary mediators in the chain of hormonal events triggered in response to stress. When these two hormones are secreted in response to stress, they

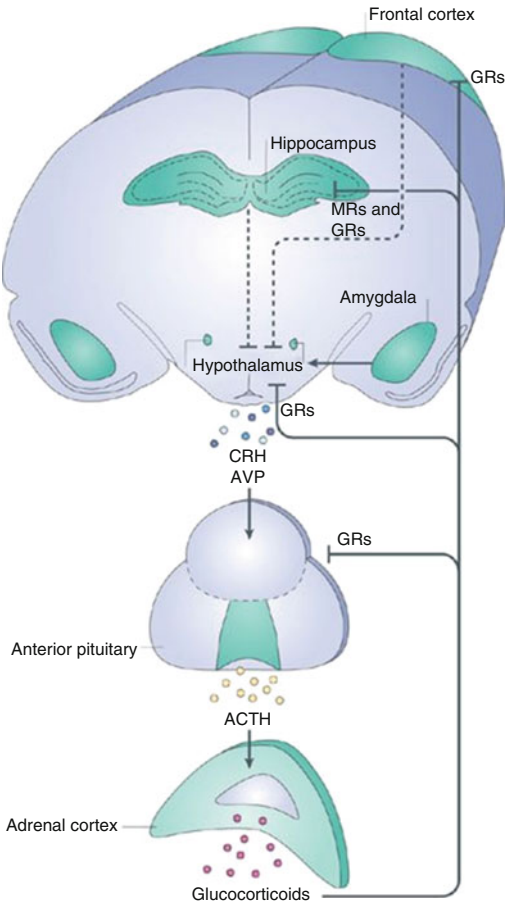

Fig. 1.1 The hypothalamic–pituitary–adrenal axis controlling the secretion of glucocorticoids (corticosterone in rats, cortisol in humans) (Reproduced with permission of the Nature Publishing Group from Lupien et al. (2009))

act on the body to give rise to the fight-or-flight response whereby one would, for instance, experience an increase in heart rate and blood pressure.

Cortisol is the primary mammalian stress hormone that functions to mobilize energy in the form of glucose metabolism at the expense of other biological systems such as reproduction, immunity, inflammation, and growth (Sapolsky et al. 1986). A wealth of animal studies have shown that this system is adaptive only when activated briefly and in proportion to the stressor magnitude, and if shut off once, it is no longer required (McEwen 1998). Given the lipophilic properties of glucocorticoids, these adrenal hormones can easily cross the blood-brain barrier and enter the brain, where they can influence brain function, behavior, and mental health by way of binding to different receptor types. Three of the most important brain areas containing glucocorticoid receptors are the hippocampus, the amygdala, and the frontal lobes, which are brain structures known to be involved in learning/memory and emotional regulation (for reviews, see Lupien et al. 1999; Lupien and Brière 2000; Lupien and Lepage 2001). Recent studies show that stress hormones can lead to impairments in attention and memory (Lupien et al. 1999; Golier et al. 2002) and emotional regulation (Maheu and Lupien 2003; Maheu et al. 2004, 2005) and are related to the development of mental health disorders (Goodyer et al. 1996, 2000; Burke et al. 2005) in vulnerable children and adults (for a review, see Lupien et al. 2009). Consequently, physiological markers of stress can provide important information on how acute and/or chronic stress can get "into the skull" and lead to cognitive deficits and/or mental health disorders.

1.2.2 The Stressor

In the past few years, a growing number of scientists started to feel the need to clarify the notion of "stressor" in both animal and human studies. For the purpose of this review, we will use the definition of stress provided by the National Scientific Council on the Developing Child at Harvard University (www.developingchild.net) because it proposes three different types of stress that have been shown to have different outcomes during development and is a definition of stress that applies to both animals and humans.

1.2.3 Types of Stress

1.2.3.1 Positive Stress

Positive stress refers to biological responses that are acute, moderate, and short-lived. These physiological responses usually have positive effects on the organism by increasing vigilance and leading to energy mobilization. These responses include increases in heart rate and blood pressure and mild elevations in cortisol or cytokine levels. There

are various events that can induce a positive stress, for example, challenges associated with meeting new individuals (humans) or new environments (animals and humans), the unpredictability of a new social position (animals and humans), or a low sense of control induced by a visit to the dentist (humans). When it occurs in the context of stable and supportive environments, positive stress is a necessary aspect of health by activating the HPA system when needed and by shutting it down when the stress is over.

1.2.3.2 Tolerable Stress

Tolerable stress represents a biological response to stress similar to the one observed for positive stress, but here, the response has the potential to lead to negative effects if the response becomes chronic. Factors that induce tolerable stress include significant threats (animals and humans), serious illness (animals and humans), a frightening injury (animals and humans), divorce, natural disaster, or acts of terrorism (humans). All of these experiences could have long-term consequences on the HPA system and the brain, but they are tolerable when they occur in a limited time period. If adequate support is provided to the individual in times of tolerable stress or if the stressor disappears, this gives the HPA axis and the brain an opportunity to recover from the potentially damaging effects of an overactive stress management system, and no long-term consequences are observed.

1.2.3.3 Toxic Stress

Toxic stress represents the strong and chronic activation of the body's stress system and the absence of a buffering protection to stop the activation of the HPA system. Factors that induce toxic stress include exposure to harsh environments (animals and humans); recurrent physical, emotional, and/or sexual abuse (humans); chronic neglect (animals and humans); or repeated exposure to violence in the community or within the family (humans). In these conditions, the persistent increase of stress hormones and the consequent altered levels of key brain chemicals produce an internal physiological state that disrupts the architecture of the developing and/or aging brain and can lead to deficits in learning, memory, and emotional regulation and eventually lead to the development of stress-related mental health disorders such as depression, post-traumatic stress disorder, or anxiety.

1.3 Brain Development in Animals and Humans

In animals that give birth to mature young (primates, sheep, and guinea pigs), maximal brain growth and most of the neuroendocrine maturation occur in utero. However, in rats, rabbits, and mice, the mother gives birth to immature young, and

most of the neuroendocrine development occurs in the postnatal period (see Kapoor et al. 2008).

In humans, the HPA axis is highly responsive at birth, but brain development is not terminated. The volume of the hippocampal formation increases sharply until the age of 2 years, while amygdala volume continues to increase slowly until the late 1920s (Giedd et al. 1996). In contrast, the development of the frontal cortex in humans is mostly observed between 8 and 14 years of age (Giedd et al. 1996). Late growth in prefrontal volumes is consistent with previous data showing that this region develops latest in terms of myelination and synaptic density in humans (Yakovlev and Lecours 1967). Consequently, the first week of the rodent's life is often equated with development of the human infant during the last trimester of gestation. Contrasting effects of prenatal and postnatal stress as a function of species are thus observed because perinatal manipulations will have different impact at different stages of development as a function of the species studied.

During animal and human aging, significant decreases in brain volumes have been reported, although most of the studies performed are cross-sectional. In humans, studies show that among men, the volume of the hippocampus starts to decrease by the second decade of life, while the decrease in hippocampal volumes is delayed until 40 years of age in women, possibly due to the protective effects of estrogen (Pruessner et al. 2000). In contrast, amygdala volume decreases at a later age, around the sixth decade of life in humans (Giedd et al. 1996), while the frontal cortex presents differential effects of age as a function of subregions. Age is significantly associated with smaller dorsolateral and inferior frontal cortex, while no age effects are reported for the anterior cingulate, frontal pole, and precentral gyrus (Tisserand et al. 2002).

1.4 Methods to Induce and Measure Stress in Animals and Humans

1.4.1 Inducing Stress

The HPA axis can be activated by a wide variety of stressors. Some of the most potent are psychological or processive stressors (e.g., stressors that involve higher-order sensory cognitive processing) as opposed to physiological or systemic stressors. Many psychological stressors are anticipatory in nature, that is, they are based on expectation as the result of learning and memory (e.g., conditioned stimuli in animals, anticipation of threats, real or implied, in humans) or species-specific predispositions (e.g., avoidance of open space in rodents, threat of social rejection, and negative social evaluations in humans).

Animal studies allow for the development of experimental protocols in which animals are subjugated to acute and/or chronic stress, and the resulting effects on the brain and behavior are studied. Experimental stressful manipulations in animals can be broadly split into prenatal and postnatal manipulations. Prenatal manipulations

involve prenatal stress, exposure to synthetic glucocorticoids, and nutrient restriction in the mother. Postnatal manipulations include maternal deprivation, modified maternal behavior, and exposure to synthetic glucocorticoids. In these protocols, the cause–effect relationship between stress and its impact on the brain can be demonstrated. In animals, stress hormones are measured in samples of feces or bile in sacrificed animals or in blood samples taken from the ear artery or tail in live animals (see Sheriff et al. 2010).

In contrast, and because of ethical issues, the cause–effect impact of stress on the brain cannot be studied in humans and most of the studies are correlational by nature. In humans, cortisol levels can be measured in individuals' natural environments (home, school, workplace, etc.) to assess basal cortisol secretion, or individuals can be exposed to an acute stressor. The most validated human stressor to this day is the Trier Social Stress Test (Kirschbaum et al. 1993). The TSST is an established and highly effective psychosocial stress paradigm used to provoke activation of the HPA axis. In summary, the task involves an anticipation phase (10 min) and a test phase, which is a 10 min public speech. The test phase is divided into a mock job interview (5 min) followed by mental arithmetic (5 min). Throughout their performance, participants are facing two confederates who act as judges and pretend to be experts in behavioral analysis. Judges observe the participants and communicate with them via an intercommunication system. The TSST has been shown to be a validated method to induce an acute increase in cortisol levels (Kirschbaum et al. 1993).

In order to assess the effects of chronic stress in humans, scientists use "experiments of nature" to obtain information about the effects of chronic exposure to early adversity on brain development and of adulthood and late-life stress effects on the brain. The intrauterine undergrowth and low birth weight are considered as indexes of prenatal stress (including malnutrition) in humans. In terms of postnatal stress, low socioeconomic status, natural disasters, maltreatment, and war are considered adverse events. In adults and older adults, studies of caregivers of those with chronic medical conditions (spouses of patients with brain degenerative disorders, parents of chronically sick children, and health-care professionals) provide a human model of the impact of chronic stress on the brain, behavior, and cognition.

1.4.2 Measuring Stress

Before the 1990s, cortisol levels were measured in blood samples. However, the invasive nature of this sampling method led to acute stress response in participants, and blood contains both the free and bound portion of cortisol. Consequently, in the mid-1990s, noninvasive methods were developed that allowed scientists to measure cortisol levels in human saliva (Kirschbaum and Hellhammer 1994). Today, the majority of studies use salivary measures of cortisol instead of blood because saliva only contains the free, unbound fraction of the hormone that is active at the glucocorticoid receptor. However, saliva measures only indicate cortisol levels produced

over short periods of time (a few days) and thus represent a physiological measure of acute stress (for a recent review, see Doom and Gunnar 2013). Recently, a new biospecimen (hair) has been tested for its capacity to provide measures of cortisol in humans over longer periods of time (Dettenborn et al. 2012). Hair grows 1 cm per month, and the interesting aspect of this new biospecimen is that cortisol levels accumulate in hair samples, providing a measure of accumulated human stress hormones over time (Kirschbaum et al. 2009). By analyzing samples of 3 cm of hair, scientists are thus able to assess human exposure to stress within the last 3 months. The hair method has been validated by many laboratories in the world and does not vary as a function of various factors such as hair dying or presence of dirt in hair (Dettenborn et al. 2012).

Although hair samples can provide measures of stress over a 3-month period, a measure of "allostatic load" provides important information on stress occurring over longer periods of time. Recent studies show that the same stress hormones (e.g., cortisol) that are essential for survival can have damaging effects on both physical and mental health if they are secreted over longer periods of time, a process called "allostatic load" (thereafter named AL; McEwen 2002). Once the primary stress hormones (glucocorticoids and catecholamines) are increased for long periods of time, this leads to dysregulation of other major biological pathways in the body and the brain (insulin, glucose, lipids, brain neurotransmitters, etc.), which then impacts various systems (cardiac, immune, neurological). We now know a great deal about the chronology of these physiological and systemic dysregulations.

By measuring various physiological parameters related to these biological pathways, we can create an AL composite score that indicates the level at which a chronic stress state has been established in any given individual (Seeman et al. 2001). At this point in time, the AL battery can include up to 17 markers of physiological dysregulation that have all been shown to be related to chronic stress states (Juster et al. 2010). For example, these markers include eight metabolic markers (total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL), fibrinogen, plasma glycosylated hemoglobin (Hb_{A1c}) reflecting glucose metabolism, insulin, aggregate systolic and diastolic blood pressure (SBP and DBP), and waist-to-hip (W/H) ratio representing abdominal adiposity) (Seeman et al. 1997a), four hormonal markers (cortisol, epinephrine, norepinephrine output, and dehydroepiandrosterone sulfate [DHEA-S]), and five immune/inflammatory markers (interleukin-6, C-reactive protein, Insulin Like Growth Factor 1, interleukin-1 beta, and TNF alpha) (for a review, see Juster et al. 2010). In general, most studies will use about 80 % of these markers (see Juster et al. 2011). For any given individual, an AL score is calculated by the number of physiological markers that are in the top (or bottom depending on the nature of the physiological variable) 25th percentile of the group to which the individual is compared. A higher AL score thus represents a higher chronic stress state (Seeman et al. 2001).

To this day, validation studies using nationally representative samples have demonstrated that AL increases throughout life but shows a steeper increase between the second and third decade of life (Crimmins et al. 2003). All of these studies show

that while none of the various physiological components of AL independently predicts various health outcomes, the composite measure of AL is significantly associated with physical and mental health outcomes, over and above each indicator taken separately (Karlamangla et al. 2002; Crimmins et al. 2003; Schnorpfeil et al. 2003; Hellhammer et al. 2004; Glover et al. 2006; Clark et al. 2007; Hasson et al. 2009; Juster et al. 2010, 2013; Chen et al. 2012; Evans and Pilyoung 2012). Moreover, the specificity of the AL score versus the well-known metabolic syndrome (visceral obesity, insulin resistance, dyslipidemia, hypertension) to assess chronic stress in humans is demonstrated: the addition of hormonal and immune markers of stress increases the predictive value of the AL model over that of the metabolic syndrome (Krumholz et al. 1994; Karlamangla et al. 2002; Reuben et al. 2003).

1.5 Effects of Stress on the Brain and Behavior: Animal Studies

1.5.1 Prenatal Stress

In animals, exposure to stress early in life has "programming" effects on the HPA axis and the brain (Barker 1991). The notion of programming involves the presence of an environmental factor that acts during a sensitive developmental period to impact on structure and function of tissues, leading to effects that persist throughout life. In rats that are born immature, prenatal stress also leads to long-term increases in HPA axis activity (Koehl et al. 1999). Controlling glucocorticoid levels in stressed dams by adrenalectomy and hormone replacement prevents these effects, indicating the importance of elevations in maternal glucocorticoids for prenatal programming of the HPA axis (Barbazanges et al. 1996).

In species that give birth to mature offspring, such as the guinea pig, a single or repeated exposure of a pregnant female to stress (Cadet et al. 1986) or to glucocorticoids (Dean and Matthews 1999) leads to increased maternal glucocorticoid secretion. Glucocorticoids can access the placenta to reach the fetus and increase fetal HPA axis activity and modify brain development (see Seckl 2008). Indeed, the placenta serves as a physical bridge between the mother and fetus. It has been suggested that maternal stress induces changes in placental phenotype that may provide a mechanism for transmitting the "memory" of early events to the fetus later in gestation, which leads to intrauterine programming of cerebral development long after the original insult (Uno et al. 1989, 1990). The placental enzyme 11βHSD2, which converts noxious maternal cortisol into benign cortisone before sending it to the fetus, plays an important role in modulating the programming effects of prenatal endogenous glucocorticoid exposure (Seckl 2001, 2004).

Maternal stress significantly reduces expression and activity in placental 11βHSD2 (Welberg et al. 2005; Mairesse et al. 2007). By contrast, downregulation of placental 11βHSD2 activity increases glucocorticoid exposure of the placenta and the fetus. Although much of maternal cortisol is metabolized as it passes through

the placenta, relatively small increases in maternal cortisol resulting from maternal stress can double fetal concentrations (Gitau et al. 1998). Furthermore, prenatal maternal stress not only increases a mother's own circulating cortisol, it also *reduces* the activity of the cortisol barrier placental enzyme, 11βHSD2, leaving the fetus less well protected (Avishai-Eliner et al. 2002) and exposed to higher levels of glucocorticoids (Austin et al. 2005; Kinsella and Monk 2009; Charil et al. 2010). Thus, the placenta plays a key role in filtering out the harmful effects of maternal glucocorticoids that reaches the fetus. Recent work has demonstrated associations between prenatal mother's stress and reduced 11βHSD2 activity (O'Donnell et al. 2012, 2014). With increased stress levels, both decreased expression and activity of the 11*β*HSD2 have been reported (O'Donnell et al. 2009, 2012). Thus, increased fetal glucocorticoids (related to increased maternal glucocorticoids) result in the dysregulation of HPA axis activity in the offspring (O'Connor et al. 2002a, b, 2003).

The type of disruption to fetal neural development may have a less significant impact than the timing in determining risk for negative outcomes (Mednick et al. 1988). Timing effects are a function of (a) the developmental stage of the fetus, (b) individual differences in maternal reactivity to stress during pregnancy, and (c) fetal exposure to glucocorticoids through placental 11βHSD2 function. Furthermore, the expression and activity of placental 11βHSD2 are regulated in a cellular and gestational age-specific manner (Pepe et al. 2001; Rosenthal et al. 2001). Worse outcomes are often associated with stress at mid-gestation (Huttenen and Niskanen 1978; Ross and Joshi 1992; Mednick et al. 1999; Watson et al. 1999; Glynn et al. 2001; O'Connor et al. 2002a; Huizink et al. 2004; Kapoor et al. 2008), a critical period for brain development (Weinberger 1995; Andreason 1999). However, some studies show third trimester effects on obstetric complications (Crandon 1979) and autism (Kinney et al. 2008). Thus, timing effects are associated with the outcome of interest.

Glucocorticoids are important for normal brain maturation, initiating terminal maturation, remodeling axons and dendrites, and acting on cell survival (Meyer 1983). Both suppressed and elevated glucocorticoid levels impair brain development and functioning. For example, administration of synthetic glucocorticoids to pregnant rats delays maturation of neurons, myelination, glia, and vasculature in the offspring, significantly altering neuronal structure and synapse formation and inhibiting neurogenesis (see Seckl 2008). Furthermore, prenatal stress decreases the number of mineralocorticoid (MR) and glucocorticoid receptors (GR) in the hippocampus of juvenile and adult rats, an effect that might occur through epigenetic effects on gene transcription (Weaver et al. 2004). Given the inhibitory role of the hippocampus on HPA activity (see Fig. 1.1), a reduced number of MR and GR induced by prenatal stress may lead to decreased hippocampal inhibition of hypothalamic sites and a resulting increased basal and/or stress-induced glucocorticoid secretion. More recent papers also show that glucocorticoids, acting via MRs, decrease resilience to stress via downregulation of mGlu2 receptors (Nasca et al. 2015).

In rhesus monkeys, prenatal treatment with the synthetic glucocorticoid dexamethasone causes a dose-dependent degeneration of hippocampal neurons, leading

to a reduced hippocampal volume at 20 months of age (Uno et al. 1990). Effects on other brain regions are also apparent. Rats exposed to stress in utero during the last week of gestation present significant decrease in dendritic spine density in the anterior cingulate gyrus and orbitofrontal cortex compared to control animals (Murmu et al. 2006). As well, prenatal exposure to glucocorticoids leads to increased adult CRH levels in the central nucleus of the amygdala, a key region in the regulation of fear and anxiety (Cratty et al. 1995).

Exposure to prenatal stress has three major effects on adult behavior: learning impairments, especially in aging rats (Vallee et al. 1999), enhanced sensitivity to drugs of abuse (Deminiere et al. 1992), and increases in anxiety-and depression-related behaviors (Vallee et al. 1997). The impaired learning performance of adult and aged rats exposed to prenatal stress is thought to be dependent upon the effects of prenatal stress on hippocampal function (Lemaire et al. 2000), while the effects on anxiety are thought to be subserved by stress-induced increased CRH in the amygdala (Cratty et al. 1995). Given that prenatal glucocorticoid exposure affects the developing dopaminergic system, which is known to be involved in reward-/drug-seeking behavior (Piazza and Le Moal 1996), it has been suggested that the enhancement of sensitivity to drugs of abuse is related to the interaction between prenatal stress, glucocorticoids, and dopaminergic neurons (Piazza and Le Moal 1996).

1.5.2 Postnatal Stress

In the postnatal period, one of the most potent stressors for pups is separation from the dam for long periods of time. Long separation periods (3 h or more each day) activate the pups' HPA axis, evidenced by increased stress-induced circulating levels of ACTH and glucocorticoids (Levine and Wiener 1988). Protracted maternal separation also reduces pituitary CRH binding sites (see Anisman et al. 1998), and low levels of maternal care reduce GR receptor number in the hippocampus (Liu et al. 1997).

The effects of maternal deprivation due to prolonged separation extend beyond the HPA axis. Early prolonged separation and deprivation in rats increase the density of CRH binding sites in the prefrontal cortex, amygdala, hypothalamus, hippocampus, and cerebellum as measured post-infancy (see Anisman et al. 1998). Given that CRH in the hippocampus is involved in stress-related loss of branches and spines (Fenoglio et al. 2006), this increase in CRH binding sites in these brain regions may confer negative effects over time. Studies have shown that the long-term effects of prolonged separation depend on the age of the pup and the duration of deprivation, with the effects noted above generally being greater when these separations occur earlier in infancy and last for longer durations (de Kloet and Oitzl 2003).

Although the rodent work provides a rich framework for conceptualizing the impact of early-life stress, the fact that the rodent brain is much less developed at

birth relative to the primate brain makes translation of the findings somewhat challenging. Nonhuman primates whose brain maturation at birth and pattern of parent–offspring relations are more comparable to humans provide an important bridge in the translation of the rodent findings. Studies in monkeys have shown that repeated, unpredictable separations from the mother (Sanchez et al. 2005), unpredictable maternal feedings (Coplan et al. 1996), or spontaneous maternal abusive behavior (Sanchez 2006) increases cerebrospinal fluid CRH concentrations and alters the offspring's diurnal activity of the HPA axis measured months or years after the period of adversity. The altered diurnal patterns of cortisol production result in *lower* than typical levels early in the morning (around wake up) and slightly higher levels later in the day, an effect that appears to reverse over time in the absence of continued, ongoing psychosocial stress (Sanchez 2006). In contrast to diurnal impacts that have not been noted in rodent studies, the effects on higher brain regions appear to be comparable to the rodent findings and include heightened fear behavior (Rosenblum et al. 2002), exaggerated startle responses (Sanchez et al. 2005), hippocampal changes such as an increase in the intensity of non-phosphorylated neurofilament protein immunoreactivity in the dentate gyrus granule cell layer (Siegel et al. 1993), and atypical development of prefrontal regions involved in emotion and behavior control (see Sanchez et al. 2001).

1.5.3 Stress in Adolescence

In rodents, the period of adolescence has three stages, a prepubescent/early adolescence period from days 21 to 34, a mid-adolescence period from days 34 to 46, and a late adolescence period from days 46 to 59 (see McCormick and Mathews 2007). Although adolescence is a time of significant brain development, there has been relatively little research on stress during this period in rodents. In adolescent rodents, HPA function is characterized by a prolonged activation in response to stressors compared to adulthood. Studies have shown that prepubertal rats have a delayed rise and a more prolonged glucocorticoid release to several types of stressors than do adult rats (Vazquez and Akil 1993), an effect that is due to incomplete maturation of negative feedback systems (Goldman et al. 1973).

In contrast to adult rats, which show a habituation of the stress response with repeated exposure to the same stressor (Girotti et al. 2006), juvenile rats present a potentiated release of ACTH and glucocorticoids after repeated exposure to stress (Romeo et al. 2006), suggesting that the HPA axis responds differently to acute and chronic stress depending on the developmental stage of the animal. Compared to animals that are stressed only in adulthood, exposure to the combination of juvenile and adulthood stress increases anxiety levels in rats (Avital and Richter-Levin 2005). Moreover, the exposure to juvenile stress results in greater HPA axis activation than a double exposure to stress during adulthood (Avital and Richter-Levin 2005), and this effect is long-lasting. These results suggest that repeated stress in adolescence may lead to greater exposure of the brain to glucocorticoids than similar experiences in adulthood.

The fact that the brain is undergoing vigorous maturation over adolescence suggests that it may be more susceptible to stressors and the concomitant exposure to high levels of glucocorticoids in adolescence than in adulthood. Consistent with this hypothesis are results showing that increased levels of glucocorticoids before (but not after) puberty alter the expression of genes for the NMDA receptor subunits in the hippocampus (Lee et al. 2003). The hippocampus of rats continues to grow well into adulthood, and studies have shown that this growth is vulnerable to chronic stress during the juvenile period (Isgor et al. 2004). Here, studies have shown that chronic, variable stress during the peripubertal juvenile period results in reduced hippocampal volume in adulthood, which is accompanied by impairments in Morris water maze navigation and delayed shutdown of the HPA response to an acute stressor (Isgor et al. 2004). Given the fact that these differences in hippocampal morphology were not evident immediately after the stressor exposure but were only evident in adulthood (Isgor et al. 2004) suggests that stress in adolescence reduces growth of the hippocampus. Finally, the effects of juvenile stress are long-lasting: adult rats exposed to juvenile stress exhibit reduced exploratory behavior and poor avoidance learning (Tsoory and Richter-Levin 2006). Moreover, studies have shown that stress in adolescence increases susceptibility to drug abuse during the adolescent period (Kabbaj et al. 2002) and in adulthood (McCormick et al. 2004).

More recent results in mice show that administration of glucocorticoids on postnatal days 2–4 significantly increases excitability of neurons of the basolateral nucleus of the amygdala in early adolescence (Koppensteiner et al. 2014). Moreover, mice treated with glucocorticoids postnatally display impaired extinction of contextually conditioned fear memory, a type of behavior in which the basolateral nucleus of the amygdala plays an important role. In contrast, exposure to the same concentrations of glucocorticoids at postnatal days 17–19 does not significantly affect basolateral amygdala electrophysiology or extinction learning in adolescence. These results suggest age-dependent effects of neonatal glucocorticoid exposure in amygdala neurons. Moreover, they provide first pieces of evidence for a negative influence of early-neonatal stress on adolescent fear memory processing.

1.5.4 Stress in Adulthood

Studies on adult stress in rodents have delineated the effects of acute versus chronic stress on the brain and behavior. The impact of acute stressors depends on the level of glucocorticoid elevations, with small increases resulting in enhanced hippocampus-mediated learning and memory, and larger, prolonged elevations impairing hippocampal function (Diamond et al. 1992). The inverted U-shaped effects of acute glucocorticoid elevations may serve adaptive purposes by increasing vigilance and learning process during acute challenges. The biphasic influence of stress might be illustrated earlier by studies showing enhancement of memory of danger by both catecholamines and glucocorticoids (Roozendaal 2000, 2002; Roozendaal and de Quervain 2005) and acquired immune response

by both catecholamines and glucocorticoids (Dhabhar and McEwen 1999; Dhabhar et al. 2012).

The mechanism underlying the acute biphasic actions of glucocorticoids on cognition involves the adrenergic system in the basolateral nucleus of the amygdala. Glucocorticoids have been shown to play a permissive role in the priming effect of the basolateral amygdala on long-term potentiation in the dentate gyrus (Vouimba et al. 2007), an effect that is mediated by a glucocorticoid-induced enhancement of noradrenergic function in the amygdala. This modulation of noradrenergic function by glucocorticoids has been linked to the enhanced memory for emotional events that occur under stress (Roozendaal et al. 2002).

In contrast to acute stress, chronic stress or chronic exogenous administration of glucocorticoids in rodents causes dendritic atrophy in the CA3 pyramidal neurons of the hippocampus (Magarinos and McEwen 1995). These changes in dendritic morphology need several weeks to develop, but they are not permanent since the CA3 dendritic atrophy is reversed to control levels by 10 days after cessation of the stressor (Conrad et al. 1999). Chronic stress in adult rats also inhibits neurogenesis in the dentate gyrus (Gould et al. 1997), an effect that is accompanied by a decrease in structural volume (McEwen 2000). Here, it is important to note that this decrease in structural volume is not associated with a change in neuron number and is not limited to the dentate gyrus (Pham et al. 2003). The morphological changes in the various hippocampal regions after chronic stress have been related to changes in spatial learning (see McEwen 2001). The spatial memory impairments induced by chronic stress are reversed following 21 days of withdrawal from stress (Luine et al. 1994). Here, it is interesting to note that in contrast to chronic/severe stress earlier in life that has long-lasting effects on the brain and behavior, adulthood stress, even when chronic, is reversed after a few weeks of nonstress. These differences between exposure to stress early in life and adulthood could be related to differences in the severity of stressors to which pups and adult rats are exposed or to differences in the development of the hippocampus at the time of exposure to stress.

Pyramidal neurons in layer II/III of the prefrontal cortex also show dendritic retraction and reduction in spine number (see Joels et al. 2007) in response to chronic stress in adulthood. This effect can be observed 24 h after a single forced swim stress (Izquierdo et al. 2006) and is related to remodeling after cessation of the stressor (Shansky et al. 2009). Volumetric analysis confirmed the morphological observations in the prefrontal cortex, as hypersecretion of glucocorticoids is associated with reduced volume of at least the right anterior cingulate cortex in rodents (Cerqueira et al. 2005). Contrary to reduction in hippocampal and frontal volumes, chronic stress in adult rodents has been shown to lead to dendritic *hypertrophy* in the basolateral amygdala (Mitra et al. 2005). Moreover, a recent study demonstrated that a single acute administration of glucocorticoids leads to dendritic hypertrophy in the basolateral amygdala 12 days later (Mitra and Sapolsky 2008). This dendritic hypertrophy in the amygdala was shown to correlate with anxiety in both the acute (Mitra and Sapolsky 2008) and chronic (Mitra et al. 2005) administration paradigms.

1.5.5 Stress in Aging

About 30 % of aged rats present basal hypersecretion of glucocorticoids correlated with memory impairments and reduced hippocampal volume (Issa et al. 1990). If a middle-aged rat is exposed for a long period to high levels of exogenous glucocorticoids, it will develop memory impairments and hippocampal atrophy (Landfield et al. 1978) similar to those observed in 30 % of aged rats. On the contrary, if a middle-aged rat is adrenalectomized (the adrenal glands secreting glucocorticoids are removed and the animal is kept alive with low doses of exogenous glucocorticoids), this will prevent the emergence of both memory deficits and hippocampal atrophy observed in old age (Landfield et al. 1981). Several groups have also found that chronic stress in aged rats can accelerate the appearance of biomarkers of hippocampal aging (e.g., frequency potentiation, synaptic excitability thresholds) and that excess endogenous or exogenous glucocorticoids induce hippocampal dendritic atrophy and inhibit neurogenesis (see Landfield et al. 2007). Finally, in aged monkeys (Kulstad et al. 2005), studies show that chronic glucocorticoid treatment can increase β-amyloid pathology similar to that reported in Alzheimer's disease.

These results have given rise to the "glucocorticoid cascade hypothesis" (Sapolsky et al. 1986) that suggests that a significant relationship exists between cumulative exposure to high glucocorticoid levels and atrophy of the hippocampus. Renamed the "neurotoxicity hypothesis" (Gilbertson et al. 2002), the explanation for the relationship between cumulative exposure to high levels of glucocorticoids and hippocampal atrophy is that prolonged exposure to these stress hormones reduces the ability of neurons to resist insults, increasing the rate at which they are damaged by other toxic challenges or ordinary attrition (Sapolsky et al. 1986). A similar neurotoxic effect may also exist in the prefrontal cortex. A study demonstrated an enhanced post-stress-induced elevation in the extracellular levels of glutamate in the hippocampus and medial prefrontal cortex of aged rats compared with young rats (Lowy et al. 1995). Increased glutamate responses after stress and perhaps other neurotoxic insults may thus increase the vulnerability of the aging brain to neuronal damage. More recent studies show that riluzole – which increases glutamate uptake through glial transporters – can protect against some of the synaptic alterations in hippocampus that are linked to age-related memory loss in rats (Pereira et al. 2014).

1.6 Effects of Stress on the Brain and Behavior: Human Studies

1.6.1 Prenatal Stress

Consistent with research in animals, data from retrospective studies on children whose mothers experienced psychological stress or adverse life events during pregnancy or in women who received exogenous glucocorticoids suggest long-term

neurodevelopmental effects on the infant (see Kapoor et al. 2008). Over the past decade, studies are increasingly zeroing in on the role of prenatal maternal mental health difficulties as conferring a specific risk to later child outcome (Glover and O'Connor 2002; O'Connor et al. 2002a, b, 2003, 2005; DiPietro et al. 2006; Barker et al. 2011a, b; Dunkel Schetter 2011; Field 2011; Dipietro 2012; Hayes et al. 2013; Graignic-Philippe et al. 2014). This work developed in part as a result of animal and human studies documenting a developmental origin of health and disease. The hypothesis of a developmental origin of health and disease (Barker 1995a, b) is based on findings from a large-scale epidemiological study suggesting that adult vulnerability to disease may be programmed during the fetal period. Consistent with this fetal programming hypothesis, there is a growing body of literature highlighting the impact of maternal mental health difficulties during pregnancy on early neurodevelopment in both animals and humans (Austin et al. 2005; Kinsella and Monk 2009; Charil et al. 2010; Buss et al. 2011; Dunkel Schetter 2011; Dunkel Schetter and Tanner 2012).

Research on humans has demonstrated that maternal anxiety and depressive symptoms in midpregnancy are associated with impaired fetal health and abdominal growth in mid- and late pregnancy (Heinrichs et al. 2002). High levels of anxiety during midpregnancy are associated with reduced gray matter volume in 6–9-year-old children (Buss et al. 2010). Furthermore, prenatal maternal stress has been associated with compromised children's language and cognitive development and with elevated emotional and behavioral problems throughout childhood (Laplante et al. 2004, 2008; King and Laplante 2005; Phillips et al. 2005; Talge et al. 2007; Kinsella and Monk 2009; O'Donnell et al. 2014). These effects persist after controlling for maternal postnatal symptoms. Elevated maternal prenatal anxiety, particularly in late pregnancy, was associated with increased emotional and behavioral problems in boys and girls, even after controlling for postnatal anxiety and depression (O'Connor et al. 2002a, b, 2003).

Studies from a project investigating the impact of a natural disaster (Project Ice Storm) in the province of Québec, Canada, have indicated that prenatal maternal reports of distress were more important than objective measures of severity of experience for predicting child cognitive, language, and emotional development outcomes (for review, see King et al. 2012). These studies, along with others (Glynn et al. 2001; DiPietro et al. 2002; O'Connor et al. 2002a, b, 2003; Glover et al. 2004), highlight how the timing of prenatal exposure to a stressful event can have a significant impact on the outcome of the child (King et al. 2009, 2012), with effects varying depending on whether exposure was during the first (early cognitive and language development, autistic traits at age 6 years), second (early cognitive and language development), or third trimester of pregnancy (e.g., motor development, emotional and behavioral difficulties) (O'Connor et al. 2002a; King and Laplante 2005; Talge et al. 2007; King et al. 2009; Buss et al. 2010).

Very few studies have measured changes in the brain as a function of prenatal stress in humans. However, a study showed that low birth weight combined with lower levels of maternal care was associated with reduced hippocampal volume in adulthood (Buss et al. 2007). Another study found that variation in maternal prenatal

stress is associated with differences in the development of white matter within limbic-frontal regions in humans (Sarkar et al. 2014). These findings are consistent with the developmental origin of health and disease (Barker 1995a, b, 2007) and suggest that early windows of opportunity may exist to prevent the deleterious effects of prenatal stress on brain development.

1.6.2 Postnatal Stress

A human equivalent of the rodent maternal separation paradigms might be studies of children who attend full-day, out-of-home day care centers. In children in day care centers, studies reported rising glucocorticoid levels over the day, an effect that is noted among toddlers relative to older preschool-aged children (Gunnar and Donzella 2002; Geoffroy et al. 2006). However, it is important to note here that the elevation of glucocorticoids observed in day care is less important than that observed in rodents and monkeys exposed to maternal separation. As well, while age accounts for most of the variation in the rise in glucocorticoid by late afternoon at child care, the quality of care is also important, with less supportive care producing larger increases, especially for children who are more emotionally negative and behaviorally disorganized (see Gunnar and Donzella 2002; Ouellet-Morin et al. 2010). So far, there is no evidence that the elevated glucocorticoid levels associated with being in day care affect development; however, children who are exposed to poor-quality child care for long hours early in development have an increased risk of behavior problems later in development (NICHD 2002).

Parent-child interactions and the psychological state of the mother also influence the child's HPA axis activity. Beginning early in the first year, when the HPA system of the infant is quite labile, sensitive parenting is associated with either smaller increases or less prolonged activations of the HPA axis to everyday perturbations (Albers et al. 2008). Maternal depression often interferes with sensitive and supportive care of the infant and young child, and there is increasing evidence that maternal depression can be a robust risk factor for a wide range of negative mental health and physical outcomes in the child (Beardslee et al. 1998; Hammen and Brennan 2003; Kim-Cohen et al. 2005; Weissman et al. 2006; Halligan et al. 2007; Hammen et al. 2008; Tully et al. 2008; Gump et al. 2009; Goodman et al. 2011; Herba et al. 2013). Symptoms of depression often co-occur with anxiety and stress, although they may be associated with differential risk to the child. Previous work has demonstrated how maternal depression over the child's preschool period can impact children's emotional development and neurodevelopment, with elevated maternal depression symptoms being associated with altered emotion-processing abilities (Szekely et al. 2014a), reduced fearfulness (Szekely et al. 2014b), elevated internalizing problems (Herba et al. 2013), and larger amygdala size at age 10 years (Lupien et al. 2011). Lupien and colleagues reported that 10-year-old children exposed to elevated symptoms of maternal depression over their lifetime demonstrated elevated levels of salivary cortisol during a stressful procedure (e.g., scanning

environment) compared to children who were not exposed to maternal depression symptoms (Lupien et al. 2011).

In contrast to elevated glucocorticoid levels in conditions of low parental care, studies in human children exposed to severe deprivation (e.g., orphanages/institutions), neglect, and abuse report *lower* basal levels of glucocorticoids, a finding that is similar to what has been observed in primates (see Gunnar and Donzella 2002). One mechanism that has been invoked to explain the development of hypocortisolism is downregulation of the HPA axis at the level of the pituitary in response to chronic CRH drive from the hypothalamus (Fries et al. 2005), while a second mechanism that has been invoked is target tissue supersensitivity to glucocorticoids (Yehuda et al. 2006). Importantly, this hypocortisolism in humans in response to severe stress may not be permanent: studies in infants in foster care show that training foster parents to provide sensitive and supportive care of foster care children results in normalization of basal glucocorticoid levels in the children after only 10 weeks of parent training (Gunnar and Quevedo 2008). This is an important information, in light with the findings of a recent study showing that exposure to early abuse during the childhood period is significantly associated with epigenetic regulation of the GR receptor in postmortem brains of suicide victims (McGowan et al. 2009), which remains modifiable subsequently.

1.6.3 Stress in Adolescence

In humans, adolescence is often considered to demarcate the period of sexual maturation (e.g., starting with menarche in girls). Interestingly, studies in human adolescents also suggest that the adolescent period is associated with heightened basal and stress activity of the HPA axis (Gunnar and Quevedo 2008; Gunnar et al., 2008). However, studies of stress in adolescence in rats cannot be translated directly to humans because the brain areas that are undergoing development differ between rats and humans: in rodents, the hippocampus continues to develop well into adulthood, whereas it is fully developed by 2 years of age in humans (Giedd et al. 1996). The frontal cortex and amygdala continue to develop in both species, but humans have larger ontogenetic bouts of development in frontal regions than do rodents.

There are indications that the adolescent human brain might be especially sensitive to the effects of elevated levels of glucocorticoids and, by extension, to stress. Recent studies on the ontogeny of MR and GR expression in the human brain show that GR mRNA levels in the prefrontal cortex are relatively high in adolescence and late adulthood compared to infancy, young adulthood, and senescence (Perlman et al. 2007). This suggests that there may be age-dependent sensitivity to GR-mediated regulation by glucocorticoids on cognitive and emotional processes that are regulated by these brain areas and various forms of psychopathology, including increased prevalence of depression and anxiety in adolescence (Dahl 2004; Paus et al. 2008). Periods of heightened stress often precede first episodes of

these disorders, raising the possibility that heightened HPA reactivity during adolescence may increase sensitivity to onset of stress-related mental disorders.

Adolescence is also a period in which the long-lasting effects of earlier stress become evident. Adolescents who grew up in poor economic conditions have higher baseline glucocorticoid levels (Evans and English 2002), as do adolescents whose mothers were depressed in the early postnatal period (Halligan et al. 2007). High early morning glucocorticoid levels that vary markedly from day to day at the transition to adolescence are not associated with depressive symptoms at that time, but predict increased risk for depression by age 16 (Halligan et al. 2007). Adolescents can also present increased AL. A cross-sectional analysis of 12–20-year-olds from National Health and Nutrition Examination Survey (NHANES) assessing multiple levels nested at the levels of individuals, families/households, and census tracts elegantly demonstrated gradients in AL levels that increased incrementally among adolescents living under low-, medium-, to high-cumulative-risk neighborhoods (Theall et al. 2012). Parental support appears to be a powerful pathway toward long-standing protection against elevated AL. An analysis of the 27-year longitudinal Northern Swedish cohort showed that high parental involvement in children's academic studies (measured by teacher and pupil ratings) at age 16 was associated with low AL at age 43, and this association remained significant while accounting for a wide range of socioeconomic confounders collected over the life course (Westerlund et al. 2012).

While early-life stress impairs the development of the hippocampus in rodents, to date there is little evidence of comparable effects in humans. Children exposed to physical or sexual abuse early in life do not exhibit reduced hippocampal volume relative to brain size as adolescents, although adults with these histories do show volume reductions (see Andersen and Teicher 2008). The adolescent finding holds even when the abused children have been selected for chronic PTSD and even though in some cases they exhibit overall reductions in brain volume (De Bellis et al. 1999).

Although early adversity has no effects on hippocampal volume in adolescence, alterations in gray matter volume and neuronal integrity of the frontal cortex and reduced size of the anterior cingulate cortex have been reported in adolescents exposed to early (and continued) adversity (Cohen et al. 2006). As well, Swartz and colleagues (Swartz et al. 2015) scanned adolescents at two timepoints, and changes in amygdala reactivity to threat-related information were assessed as a function of family history of depression and severity of stressful life events. Results showed that heightened amygdala reactivity emerges during adolescence, prior to the development of depression, as a function of familial risk or, in the absence of familial risk, stressful life events (Swartz et al. 2015).

Together, these results suggest that the frontal cortex and the amygdala, which still continue to develop during adolescence, might be particularly vulnerable to the effects of stress occurring during adolescence. In contrast, the hippocampus, which develops mostly in the first years of life, might be less affected by exposure to adversity in adolescence.

1.6.4 Stress in Adulthood

In humans, studies of the effects of acute stress confirm animal studies and report the presence of an inverted U-shaped function between glucocorticoid levels and cognitive performance (see Lupien and McEwen 1997). Yet, contrary to animal studies in which most laboratory tests for learning and memory involve a fear and/or emotional process (Roozendaal 2000), tests of learning and memory in humans can differentiate the effects of glucocorticoids on the processing of neutral versus emotional information. What most studies have shown at this point is that acute increases of glucocorticoids significantly increase memory for emotional information, while a similar increase in glucocorticoids impairs the retrieval of neutral information (see Lupien et al. 2005).

Increased markers of AL have also been reported as a function of socioeconomic status in adulthood. Here, socioeconomic factors have been shown to affect AL differently according to one's sex and age. A recent study of 199 healthy Montreal workers revealed that higher occupational status was associated with higher AL in men, whereas the reverse occurred for women (Juster et al. 2013). As has been substantiated in primate research that parallels socioeconomic inequalities in humans (Sapolsky 2005), males at the top of the hierarchy might be challenged by unique sets of adversity that exacerbate rather than attenuate physiological functioning.

In terms of chronic stress in normal adult humans, only a few reports suggest an association between exposure to chronic stress and reduced hippocampal volume in individuals not suffering from mental health disorders (for a review, see Lupien et al. 2007). Yet, a recent study reported that low self-esteem, which is a potent predictor of increased reactivity to stress in humans (Pruessner et al. 2004), is significantly associated with reduced hippocampal volume (Pruessner et al. 2005).

Most of the studies of chronic stress effects on the adult human brain have concentrated on either stress-related psychopathologies or on the impact of early-life stress on adult psychopathology. A large number of studies now report elevated basal glucocorticoid levels in some forms of depression (Burke et al. 2005), whereas reduced concentrations of basal glucocorticoids are reported in PTSD (Yehuda et al. 2005; but see Meewisse et al. 2007). Given that low glucocorticoid concentrations seem to develop in early childhood in response to neglect or trauma, it may be possible that low cortisol predicts vulnerability to PTSD developing in response to adult trauma.

Studies of adults with depression or PTSD pursuant to childhood abuse also reveal hyperreactivity of the HPA axis in abused–depressed individuals (Heim et al. 2000) and hypoactivity in those with PTSD (Yehuda et al. 2005). These changes have been associated with CRF-induced "escape" of glucocorticoid secretion from dexamethasone suppression among abused–depressed adults (Heim et al. 2008), suggesting impaired glucocorticoid feedback of the HPA axis under conditions of increased hypothalamic drive. Thus, a decreased capacity of glucocorticoids to inhibit HPA axis when stimulated may further accentuate central nervous system responses to stressors. Going along with this suggestion, increased cerebrospinal

fluid CRF levels have been reported in individuals with perceived childhood stress (Carpenter et al. 2004) and childhood abuse (Heim et al. 2001; Bradley et al. 2008).

Decreased hippocampal volume and function are landmark features of depression and PTSD (Videbech and Ravnkilde 2004; Smith 2005). Vythilingam et al. (2002) found that a smaller hippocampus in women with major depression was associated with experiences of childhood trauma, whereas depressed women without such trauma had hippocampal volumes similar to healthy controls, supporting the notion that certain brain changes seen in patients with depression or PTSD may represent markers of vulnerability for the disorder rather than markers of the disorder itself. This finding is in line with results from a twin study of Vietnam veterans by Gilbertson and colleagues (Gilbertson et al. 2002) showing that decreased hippocampal volume is not a consequence of combat exposure or PTSD, but is also present in unexposed co-twins, thus reflecting a preexisting risk factor for PTSD that could be genetic or rooted early in life.

1.6.5 Stress in Aging

Results similar to animal studies have been obtained in aged humans. Normally aging humans exhibit higher mean diurnal values of cortisol than younger individuals (Raskind et al. 1994). A longitudinal study also found that elevated plasma glucocorticoid levels over 6 years in older adults correlate with reduced hippocampal volume and memory impairments (Lupien et al. 1998). Given that aged individuals suffering from Alzheimer's disease present both memory impairments and hippocampal atrophy, studies have assessed basal glucocorticoid levels in this population. It has been found that basal glucocorticoid levels are increased in patients with Alzheimer's disease when compared to controls (Giubilei et al. 2001). As well, chronic glucocorticoid treatment has been shown to worsen cognition in people with Alzheimer's disease (Aisen et al. 2000).

The frontal lobe also seems to be sensitive to glucocorticoid effects during human aging. Using a novel in vitro postmortem tracing method on human brain slices, Dai et al. (2004) found a "bell-shaped"-type effect of glucocorticoids on axonal transport in prefrontal neurons. In most cases, a stimulating effect at low concentrations, and a depressing effect at high concentrations, was found. Given that axonal transport plays a crucial role in neuronal survival and function, these results suggest potential negative effects of glucocorticoid on prefrontal cortex neurons' survival and/or function. In a more recent study of 388 middle-aged male twins who were 51–59 years old, Kremen et al. (2010) reported small but significant negative phenotypic associations between glucocorticoid levels and thickness of left dorsolateral (superior frontal gyrus, left rostral middle frontal gyrus) and ventrolateral (pars opercularis, pars triangularis, pars orbitalis) prefrontal regions and right dorsolateral (superior frontal gyrus) and medial orbital frontal cortex. Further bivariate genetic analyses showed that these associations were primarily accounted for

by shared genetic influences; that is, some of the genes that tended to result in increased glucocorticoid levels also tended to result in reduced prefrontal cortical thickness (Kremen et al. 2010).

Older adults have been shown to be highly sensitive to AL. The MacArthur Study of Successful Aging used to validate the AL model consistently found that higher AL predicts incidences of cardiovascular disease, physical declines, cognitive impairments, and all-cause mortality (Seeman et al. 1997b, 2001, 2004b). Interestingly, even in this representative group of successful agers from diverse race/ethnicity and socioeconomic status, lower education was a powerful contributor to higher AL levels (Seeman et al. 2004a). This patterning provides evidence of health gradients found not only between but also within socioeconomic strata (Adler et al. 1994).

Likewise, a cross-cultural study of Taiwanese elders from the Social Environment and Biomarkers of Aging Study (SEBAS) revealed that higher AL and lower education and income were independently associated with health status (Hu et al. 2007). By combining the Taiwanese and MacArthur cohorts, it was found that lower SES (lower education, occupational status, and finances) together with greater social challenges (e.g., recent widowhood, high family demands) relates to higher AL (Weinstein et al. 2003). This suggests that SES and adversity might synergize among different older adults from North America and Asia.

Some of the most compelling epidemiological evidence in support of the AL model has come from the large-scale American National Health and Nutrition Examination Survey (NHANES). Findings show that AL mitigates the effects of education and income gradients predictive of ischemic heart disease and periodontal disease (Sabbah et al. 2008). Tracking about 15,000 participants from the same cohort over 6 years, it was shown that those living in poverty show sharper increases in AL up until middle age and then plateau around ages 70 and above (Crimmins et al. 2003). Strikingly, those with high AL have a life expectancy that is 6 years shorter than those with low allostatic load but with similar poverty status and matched for sex (Crimmins et al. 2009).

1.7 Models Developed to Explain the Effects of Stress on the Brain and Behavior

Several models have been developed to explain how childhood adversity affects physiological systems sensitive to the environment (Dodge et al. 1990; De Bellis 2001; Krieger 2001; McEwen 2003; Essex et al. 2006; Rutter 2006; Ouellet-Morin 2008; Gilbert et al. 2009; Lupien et al. 2009; Shonkoff et al. 2009; Sameroff 2010). Two of these models ("cumulative damage hypothesis" and "sensitive period hypothesis") concentrate on stress during early development, and here, three periods are usually considered: early childhood (0–5 years), middle childhood (6–12 years), and adolescence (13–18 years). These periods match distinct stages during which children acquire increased autonomy and physical, cognitive, and emotional

maturity while navigating their way across different socialization contexts. By and large, these models recognize that experiences occurring after the first years of life may alter the course of development (Cicchetti and Rogosch 2002). The last model (the "life cycle model of stress") takes a larger developmental approach and suggests that stress, via activation of the HPA axis and the release of glucocorticoids, has enduring effects on the brain, with the greatest impact on brain structures that are still developing at the time of the stress exposure (in young individuals) and on brain structures that are showing age-related changes (in adult and aged individuals).

1.7.1 The Cumulative Damage Hypothesis

The cumulative damage hypothesis posits that accumulating exposure to adversity, both in terms of quantity or duration, overwhelms the physiological systems designed to support adaptation to stress, increasing vulnerability to disease in the long run (Sameroff 2010). Repeated exposure to adversity triggers a cascade of physiological changes such as cortisol secretion that may, over time, alter physical and mental health (McEwen and Stellar 1993). According to the cumulative damage hypothesis, findings indicate that the more severe and persistent the adversity is, the more likely are physiological disruptions and risks for mental health (Nagin and Tremblay 1999; Flaherty et al. 2006; Whitaker et al. 2006; Green et al. 2010). Similar "dose-response" relationships have been found in research on the HPA axis (Evans et al. 2007; Ouellet-Morin et al. 2011a, b; Rogosch et al. 2011). To the best of our knowledge, the cumulative damage hypothesis has not been tested on the proposed physiological mediators in a cohort of adolescents for whom adverse experiences were documented since birth.

1.7.2 The Sensitive Period Hypothesis

According to the second model, the sensitive period hypothesis, adversity changes physiological set points provided they occur during a sensitive period (Shonkoff et al. 2009). The general assumption is that the earlier the adversity, the greater and resistant these changes become due to the brain's immaturity and sensibility to environmental tuning (Fries et al. 2005). If true, adversity would differentially affect the mediators depending on when it occurs (Weber et al. 2008). Despite the sharp increase in psychiatric illnesses in adolescence, a paucity of research examined the relative impact of early versus middle childhood adversity on the physiological mediators (Andersen 2003; Lupien et al. 2009; Neigh et al. 2009). Past research has primarily targeted the family, including harsh parenting practices, abuse, and parents' psychopathology (Gunnar and Quevedo 2008).

This corpus of research has greatly advanced our understanding of the physiological mechanisms by which childhood adversity may increase later mental health

difficulties. Less attention has been given to adversity occurring in peer contexts despite its documented impact on mental health (Arseneault et al. 2010). From a developmental perspective, this gap in knowledge is important because familial and peer contexts may differentially affect the proposed mediators across childhood (e.g., peer adversity may have a greater impact on the HPA axis in middle childhood, when peer relations become more important in the children's lives), as suggested in a preliminary report (Teicher et al. 2010). The study of the impact of adversity in peer contexts on the HPA axis and DNA methylation is still at its infancy, though recent findings published by Ouellet-Morin and colleagues support this as a possibility (Ouellet-Morin et al. 2011a).

1.7.3 The Life Cycle Model of Stress

Ouellet-Morin (2008) and Lupien et al. (2009) have proposed that the consequences of chronic stress and trauma at different life stages depend on which brain regions are developing or declining at the time of exposure (see Fig. 1.2). Stress in the prenatal period affects development of the hippocampus, prefrontal cortex, and amygdala, leading to well-substantiated *programming effects*. From the prenatal period onwards, all developing brain areas are sensitive to the effects of stress hormones; however, some areas undergo rapid growth during key critical windows. For example, from birth to 2 years of age, the developing hippocampus is extremely vulnerable to glucocorticoid surges. By contrast in late childhood (10 years old), the amygdala appears to be more vulnerable to hypertrophy in the context of exposure to maternal depression (Lupien et al. 2011). During adolescence, the amygdala continues to develop until young adulthood and finally the frontal lobe undergoes important maturation. Consequently, stress exposure during this transition into adulthood can have major effects on the frontal cortex. Studies show that adolescents are highly vulnerable to stress because of pubertal changes in gonadal

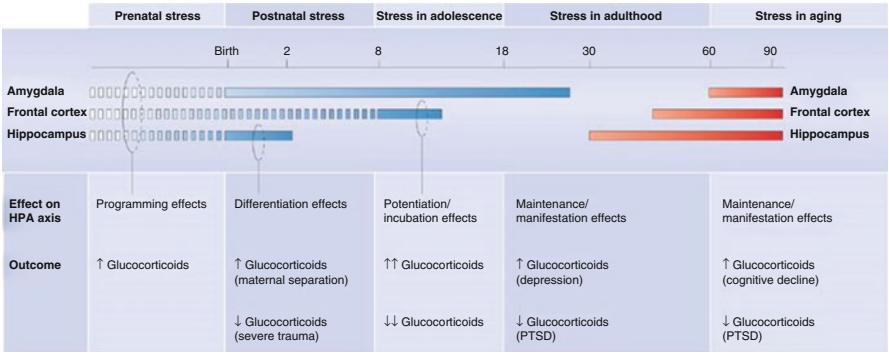

Fig. 1.2 The life cycle model of stress (Reproduced with permission of the Nature Publishing Group from Lupien et al. (2009))

hormones and sensitivity of the HPA axis that can persist into adulthood as *potentiation/incubation effects*. In middle and older adulthood, the brain regions that undergo the most rapid decline as a result of senescence are once again highly vulnerable to the effects of stress hormones. This leads to the manifestation of incubated effects from earlier life on the brain referred to as *maintenance effects* (Lupien et al. 2009).

Lifelong brain perturbations can diminish an individual's ability to adapt. This leads to subtle recalibrations in stress responsivity that could be used to detect disease trajectories (McEwen 1998). According to the life cycle model of stress, regional volumes of these neurological structures in conjunction to biological signatures (e.g., hypercortisolism or hypocortisolism) can be used to predict differential risk profiles for specific psychopathologies (e.g., depressive or PTSD) in adulthood as well as inform when certain traumas might have occurred in early life (Lupien et al. 2009). This hypothesis complements the AL model. Given that direct measurement of central nervous system substrates is costly and potentially invasive, indirect assessment using peripheral biomarkers routinely collected in blood draws could be used to measure AL algorithms (Juster et al. 2012). By this same logic, developmental trajectories that promote resilience at a neurological level could also be identified.

1.8 Epigenetics and Stress

In the last decade, growing evidence indicates that the genome is not as immutable as previously thought but rather responds dynamically to the environment (Bagot and Meaney 2010). Epigenetics refers to a series of modifications in the chemistry of the DNA that has the potential to alter expression without changing its sequence. The epigenome is conceptualized as an interface between the environment and the genome. In addition to the intrauterine environment (Waterland and Jirtle 2003; Heijmans et al. 2008; Tobi et al. 2009), recent evidence suggests that postnatal social environments can also alter epigenetic signals (Szyf et al. 2008, 2009b) which represents a biologically plausible mechanism by which childhood adversity may have long-lasting influences on health (Jirtle and Skinner 2007; Tsankova et al. 2007; Feinberg 2008; Shonkoff et al. 2009; Szyf 2009a; Tremblay 2010; Roth and Sweatt 2011).

To date, most empirical findings supporting this claim come from animal models of early-life stress. In a series of innovative experiments, it was shown that maternal care received by rat pups during the first week of life changes DNA methylation of the GR gene (*NR3C1*) promoter in the hippocampus, GR expression, and stress reactivity in adulthood, suggesting persistent changes (Weaver et al. 2004, 2006; Meaney and Szyf 2005). Additional studies have extended these findings to other genes regulating stress and neurodevelopment, such as the arginine vasopressin and brain-derived neurotrophic factor (*BDNF*) genes (Murgatroyd et al. 2009; Roth et al. 2009). Similar findings have been reported in nonhuman primates (Kinnally et al. 2010; Provencal et al. 2012).

Very little research has investigated the epigenetic remodeling following early adversity in humans. Preliminary reports suggesting higher methylation of the GR gene and *SERT* in adults with a history of childhood abuse or low SES (measured retrospectively) are consistent with that hypothesis (McGowan et al. 2009; Beach et al. 2010, 2011; Labonte et al. 2012). Similarly, differential clustering patterns of DNA methylation are reported across the genome in children raised in institutional care versus controls (Naumova et al. 2011). Birth cohorts with prospective measures of adverse life conditions have recently contributed to clarify the temporal sequence of these associations and have shown distinct DNA methylation profiles in adults exposed to different socioeconomic conditions during their childhood (Borghol et al. 2012; Tehranifar et al. 2013).

To the best of our knowledge, only three human longitudinal studies have explored the associations between early adversity and DNA methylation using a wide range of adversity measures collected repeatedly (Essex et al. 2011; Booij et al. 2012; Ouellet-Morin et al. 2013). The first comprises 109 adolescents exposed to maternal stress during their first year who showed elevated DNA methylation in 139 sites located across the genome (FDR < 5 %) (Essex et al. 2011). The second study showed that bullied twins had higher *SERT* DNA methylation compared to their non-bullied MZ co-twins, a difference that cannot be attributed to the children's genetic makeup or shared familial environments because of the study design (Ouellet-Morin et al. 2013). The third study tested whether perinatal adversity in humans has a long-term impact on brain serotonin neurotransmission in adulthood. Twenty-six healthy males, recruited from a 27-year longitudinal study, underwent a positron emission tomography scan with the tracer alpha-[^{11}C]methyl-L-tryptophan (^{11}C-AMT) as an index of serotonin synthesis capacity. The results showed that lower ^{11}C-AMT trapping in the medial orbitofrontal cortex and hippocampus was predicted by birth complication, maternal smoking, and low birth weight (Booij et al. 2012).

1.9 Opening Windows of Opportunities: Intervening Early to Change Developmental Trajectories of Stress

What can be done to remediate the effects of chronic stress, as well as the biological embedding (Hertzman 1999) associated with early-life adversity? As we have seen above, genetic factors interact seamlessly with environmental influences not only during development but also in adult life, leading to the newer meaning of "epigenetics." Thus, at each stage of development, there is no "going back," and a new set of possibilities emerges that offer opportunities for epigenetic influences. Interventions will not, therefore, "reverse" developmental events but rather produce compensatory mechanisms (Caldji et al. 1998). Indeed, development never ends, and adolescents, young adults, mature, and aging individuals continue to show the results of experiences, including opportunities for redirection of unhealthy tendencies through a variety of interventions (Halfon et al. 2014).

Interventions to foster compensatory mechanisms may involve pharmaceutical, as well as behavioral, or "top-down" interventions (e.g., interventions that involve integrated CNS activity). These include cognitive-behavioral therapy, physical activity, and programs that promote social support, social integration, and developing meaning and purpose in life (Ganzel and Morris 2011; McEwen and Gianaros 2011). More targeted interventions for emotional and cognitive dysfunction may arise from fundamental studies of such developmental processes as the reversal of amblyopia and other conditions by "releasing the brakes" that delay structural and functional plasticity (Vetencourt et al. 2008). It should be noted that many of these interventions that are intended to promote plasticity and slow decline with age – such as physical activity and positive social interactions that give meaning and purpose – are also useful for promoting "positive health" and "eudaimonia" (Ryff and Singer 1998; Singer et al. 2005) independently of any notable disorder and within the range of normal behavior and physiology.

Moreover, interventions directed toward changing physiology and brain function may be useful when adaptation to a particular environment has resulted in an individual who then chooses, or is forced, to adapt to a different, e.g., more or less threatening or nurturing, environment. A powerful "top-down" therapy (e.g., an activity, usually voluntary, involving activation of integrated nervous system activity, as opposed to pharmacologic therapy which has a more limited target) is a regular physical activity, which has actions that improve prefrontal and parietal cortex blood flow and enhances executive functions (Colcombe et al. 2004). Moreover, regular physical activity, consisting of walking an hour a day, 5 out of 7 days a week, increases hippocampal volume in previously sedentary adults (Erickson et al. 2009). This finding complements work showing that fit individuals have larger hippocampal volumes than sedentary adults of the same age range (Erickson et al. 2009). It is also well known that regular physical activity is an effective antidepressant and protects against cardiovascular disease, diabetes, and dementia (Babyak et al. 2000; Snyder et al. 2010). Moreover, intensive learning has also been shown to increase volume of the human hippocampus (Draganski et al. 2006).

Social integration, social support, and finding meaning and purpose in life are known to be protective against AL (Seeman et al. 2002) and dementia (Boyle et al. 2010). Programs such as the Experience Corps, which promotes both cognitive adaptations along with increased physical activity, have been shown to slow the decline of physical and mental health and to improve prefrontal cortical blood flow in a similar manner to regular physical activity (Fried et al. 2004; Carlson et al. 2009).

It is important to reiterate that successful behavioral therapy, which is tailored to individual needs, can produce volumetric changes in both prefrontal cortex in the case of chronic fatigue (de Lange et al. 2008) and in the amygdala, in the case of chronic anxiety (Holzel et al. 2010). This reinforces two important messages: (1) that plasticity-facilitating treatments should be given within the framework of a positive behavioral or physical therapy intervention and (2) that negative experiences during the window may even make matters worse (Castren and Rantamaki 2010).

In order to prevent the deleterious impact of stress on the developing brain, it is essential to intervene early in life. Given the adverse effects of stress on well-being and its potential implication in the vulnerability to adolescent depression, the need to provide children and adolescents with the skills to develop ways to manage and cope with stress is paramount. However, while research has increasingly examined the effects of stress management techniques in adults (Murphy 1996; Edwards et al. 2003; Gaab et al. 2003, 2006; Richardson and Rothstein 2008) and children (for a review, see Pincus and Friedman (2004), there is a paucity of research on how stress management programs (educational or psychological) apply in adolescents.

Three types of prevention programs have been developed to target different populations. Universal programs are usually presented to all individuals regardless of symptoms and are often designed to build personal competence and/or enhance general mental health (Barrett and Turner 2001). Selective programs are presented to individuals who are at risk of developing a mental health problem as a function of particular risk factors, while indicated programs are delivered to individuals who present mild or severe symptoms of a mental health disorder (Donovan and Spence 2000).

When dealing with adolescents, the school system has been identified as an ideal setting for the implementation of prevention programs (Masia-Warner et al. 2005) because this setting offers the unequaled opportunity to reach all adolescents and thus avoid the selection bias of clinically referred samples defined on the basis of mental health problems. This serves to reduce and alleviate many of the common barriers to treatment in the community such as those related to time, location, stigmatization, transportation, and costs (Barrett and Pahl 2006; Masia-Warner et al. 2006). Because universal interventions have the advantage of avoiding the stigma of singling out individuals for treatment (Rapee et al. 2006; Sheffield et al. 2006), some universal programs on stress have been developed for adolescents. For example, the *Gatehouse Project* was created to reduce stressors in the environment by creating a more inclusive classroom environment with a focus on improving interpersonal bonds (Patton et al. 2000, 2006). Similarly, the *Transition Club Project* was developed to help students gradually acclimate to the secondary school environment through pre-transition exposure (Humphrey and Ainscow 2006). While most of the stress management programs can be time consuming, review of intervention programs in school settings show that even brief school-based intervention programs can have significant effects on stress management skills in children and adolescents (Pincus and Friedman 2004).

Although some universal programs are reported to lead to significant psychological changes in the group of adolescents exposed to them, many reports indicate that most intervention programs exert a real, immediate, and measurable effect only for a subsample of participants. For example, studies dealing with prevention of depressive symptoms in children and adolescents report that certain individual characteristics (e.g., age, sex, ethnicity, cognitive ability) moderate the effects of preventive interventions on depression (Horowitz and Garber 2006). In a study by Spaeth et al. (2010), it was also shown that a universal school-based life skills program against substance misuse exerted a differential effectiveness for young

adolescents according to their alcohol use, namely, trajectories characterized by late-childhood risk factors such as temperament, self-worth, and social problems with peers (Spaeth et al. 2010). Another recent study report that selective school-based alcohol prevention programs targeting youth with personality risk factors for addiction can reduce substance use and misuse in those with elevated personality profiles (anxiety sensitivity, hopelessness, impulsivity, and sensation seeking) (Conrod et al. 2013).

The vast majority of programs for stress management developed to this day for adolescents are based on the appraisal model of Lazarus and Folkman (Lazarus and Folkman 1984). Although many of these programs have revealed beneficial effects in adolescents, it is interesting to note that none of them have been developed in line with discoveries made in the last 35 years in the field of psychoneuroendocrinology, a field of research that measures the causes and consequences of physiological reactivity to psychological stress in humans. Moreover, and as summarized by Adam and colleagues (2008) in a recent review of the literature, there is no study to this day that assessed whether physiological measures of stress respond as efficiently to prevention programs as psychological measures do. Yet, as we have seen in the present review of the literature, it is the physiological response to stress that can get under the skin and into the skull. This exerts a long-lasting influence on stress perception and coping and, in turn, underlines vulnerability to various mental health problems in adolescents (for a review, see Lupien et al. 2009).

Psychoneuroendocrine studies performed in the last three decades in humans show that there are four situational determinants that activate the HPA axis in humans, namely, novelty (N), unpredictability (U), threat to personality (T), and a sense of low control (S) (Mason 1968a; Dickerson and Kemeny 2004). The acronym NUTS can be used for the purposes of knowledge translation. In a previous study performed in 406 children and adolescents, we reported that the transition from Grade 6 (elementary school) to Grade 7 (high school) is associated with a significant increase in cortisol levels in adolescents from both low and high socioeconomic strata (Lupien et al. 2001). This finding suggested that this life transition may represent a significant stressor in the life of adolescents (for a review, see Lupien et al. 2001). Transition to high school has been reported to be associated with negative outcomes including poorer attendance, declines in grades, newly emerging disciplinary problems, and new feelings of alienation or social rejection (Moyer and Motta 1982) as well as a decline in a sense of school belongingness and an increase in depressive symptoms (Newman et al. 2007).

Based on these findings, we developed the *DeStress for Success Program*© to expand youth awareness and scientific knowledge on identifying and coping with stress (Lupien et al. 2013). The uniqueness of the *DeStress for Success Program*© lies primarily in its theoretical framework rooted in psychoneuroendocrinology. Specifically, stress is recognized and deconstructed based on the four important "NUTS characteristics" reported to lead to significant activation of the HPA axis (Mason 1968b; Dickerson and Kemeny 2004). Secondly, the program explains the stress response and ways to use the body to stop it. Finally, it is a relatively short program and easy to implement in school settings.

We evaluated the capacity of the program to decrease cortisol levels and/or depressive symptoms in 504 adolescents making the transition to high school. Adolescents of one school were exposed to the *DeStress for Success Program*©, while adolescents from the other school served as controls. Salivary cortisol levels and depressive symptomatology were measured before, immediately after, as well as 3 months after exposure to the program. Measures of negative mood were obtained at baseline in order to determine whether adolescents starting high school with specific negative moods were differentially responsive to the program. The results show that only adolescents starting high school with high levels of anger responded to the intervention with a significant decrease in cortisol levels. Moreover, we found that adolescents who took part in the intervention and showed decreasing cortisol levels following the intervention (responders) were 2.45 times less at risk to suffer from clinical and subclinical depressive states 3 months post-intervention in comparison to adolescents who showed increasing cortisol levels following the intervention (nonresponders). This study provides the first evidence that a school-based program on stress is effective at decreasing cortisol levels and depressive symptomatology in adolescents making the transition to high school. Moreover, this has helped us further understand which adolescents are sensitive to the program and what are some of the characteristics of these individuals. More importantly, these results showed for the first time that transferring scientific knowledge from the laboratory to the public *can* lead to significant changes in physiology and mental health.

1.10 Conclusion

Animal and human studies performed over the last two decades have provided a wealth of results showing the negative effects of chronic exposure to stress and/or adversity on the developing brain. Yet, stress is not and should not be considered solely as a negative concept. Stress is a physiological response that is necessary for survival of the species. The stress response that today can have negative consequences for brain development and mental health may have conferred the necessary tools to our ancestors in prehistorical times to survive in the face of predators. Studies of modern individuals who have developed resilience in the face of significant adversity should inform us about the physiological and psychological mechanism(s) at the basis of vulnerability or resilience to stress. Understanding the mechanisms underlying vulnerability and resilience to stress, which are rooted in genes and modulated by the family environment, is extremely important if one wants to provide interventions early enough to help individuals who may be most susceptible. This is essential, because this review has clearly revealed the potential for early intervention to prevent the deleterious effects of stress on brain, behavior, and cognition. After more than 60 years of research on the negative effects of stress on the brain, it is now time to turn our attention toward the potential positive impact of early interventions on brain development. These results could help develop social policies that treat the problem of early-life stress at its root, e.g., directly into the family home.

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Chapter 2
Dynamic Regulation of Chromatin Modification and Transcription by GR and the Steroid Receptors

Charlotte L. George and Becky L. Conway-Campbell

Abstract The regulation of gene expression needs to be a highly dynamic process to enable cells to rapidly respond to changing physiological environments and maintain optimal physiological function. Understanding how quickly transcription factors interact with DNA; how these interactions effect and are influenced by levels of DNA methylation and histone modifications; and how long these interactions persist for has led to new insights into the fundamental molecular processes underlying gene expression. This chapter highlights the importance of several different timeframes that should be considered when understanding the molecular mechanics underlying gene regulation. These timeframes range from long-term preprogramming of transcription factor binding sites to the fast molecular dynamics of DNA–protein interactions that occur on a scale of seconds to minutes. In particular we focus on the steroid receptor class of nuclear receptors, endocrine-activated transcription factors that exhibit highly dynamic transcriptional interactions. We specifically highlight the complexity and importance of transcriptional dynamics in the glucocorticoid system and how disruption of these dynamics may be important in disease.

Keywords Ultradian rhythm • Circadian rhythm • Transcription • Pulsatility • Gene regulation • Nuclear receptor

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2.1 Introduction

Traditionally epigenetic modifications are defined as mitotically or meiotically heritable factors affecting gene expression that are not encoded in the DNA sequence. Whilst the majority of this book focuses on epigenetic changes characterised by chromatin modification that can persist over long periods of time, it is also essential to remember that chromatin structure, DNA methylation and histone modifications can change states within minutes in a highly dynamic manner. In fact the glucocorticoid receptor's (GR's) ability to sense rapidly changing levels of glucocorticoid secretion and quickly alter chromatin structure and the expression of glucocorticoid target genes is fundamental to its role mediating the body's stress response, and its role in mediating normal physiological function in healthy unstressed individuals.

This chapter highlights the importance of a number of different timeframes, rhythms and dynamics of transcription factor binding and chromatin modification underlying gene expression. These are not just specific to the glucocorticoid system and GR but are also exhibited (although in subtly different manners) by other transcription factors, including many of the steroid receptors closely related to GR in the nuclear receptor family. We review findings that suggest GR and other steroid receptors interact with chromatin and epigenetic marks in a highly dynamic manner, enabling them to direct gene-specific, tissue-specific and context-specific transcriptional responses that are essential for normal biological function and healthy biological states. Finally we focus in particular on glucocorticoids and the stress system, highlighting the importance of glucocorticoid secretion patterns in the regulation of transcription during health and disease.

2.2 Steroid Receptors and DNA Binding

Previous chapters have highlighted the importance of modulating GR activity and how this can affect the response to glucocorticoid hormones released during stress. One reason why changes in stress hormones can have such diverse and adverse effects (ranging from inflammatory suppression to metabolic disorder or depression) is that upon binding to glucocorticoids, GR acts as a potent transcriptional regulator, directly interacting with DNA to regulate the transcription of hundreds of genes in a tissue-specific manner. It is this ability of GR to bind directly to activating ligands, translocate to the nucleus and modulate gene expression or repression by interacting with DNA promoter and enhancer regions to recruit histone modifiers, chromatin remodelling factors, transcriptional machinery and RNA polymerase that has made GR and other closely related steroid receptors a popular and simplistic model for the study of eukaryotic transcription. Thus, compared to many transcriptional systems, much is known regarding the molecular mechanisms underlying transcriptional regulation by many of the steroid hormones and their receptors in the nuclear receptor

family. However, among this receptor family, the effects of some members (such as GR and the oestrogen receptor (ER)) have been more thoroughly characterised than others.

GR, ER, the mineralocorticoid receptor (MR), androgen receptor (AR) and progesterone receptor (PR)) are all ligand-activated transcription factors in the steroid hormone subfamily of nuclear receptors. These receptors share closely related structures, comprising of the N-terminal domain (NTD), the DNA binding domain (DBD) and the C-terminal ligand-binding domain (LBD). These structures are highly tailored to their role as hormone-dependent ligand-activated transcription factors and enable ligand-activated steroid receptors to interact rapidly with DNA at distal enhancer or gene-proximal promoter regions to recruit chromatin remodelers, coactivator and corepressor complexes at specific gene regions in a highly ordered and sequential manner (Firzlaff and Diggelmann 1984; Cordingley et al. 1987; Shang et al. 2000; Métivier et al. 2003) to regulate the expression of hundreds of target genes (Kininis et al. 2007; Krum et al. 2008; John et al. 2009; Chen et al. 2010). Whilst steroid receptors are known to interact with DNA in several ways to regulate transcription, (typically by either binding to sequence-specific palindromic DNA sequences termed response elements or by interacting or 'tethering' with other DNA-bound transcription factors,) significant advances in high-throughput sequencing technology and imaging studies have shed light on the dynamics and mechanisms underlying these protein–DNA interactions and the consequent changes to chromatin structure.

2.2.1 Chromatin Accessibility and Steroid Receptor Binding

In order to put these more recent advances in context, it is first important to understand some of the early fundamental discoveries in nuclear receptor research. Due to difficulties in identifying steroid receptor-mediated genes, early studies relied upon characterisation of a few well-studied candidate genes. In the 1980s and 1990s, seminal studies studying the binding of GR to multiple glucocorticoid response elements (GREs) to a stably integrated *mouse mammary tumour virus* (MMTV) promoter and also the endogenous *tyrosine aminotransferase* (TAT) gene suggested that GR was able to act as a 'pioneer protein'. In this capacity GR was found to bind directly to GREs to induce 'de novo' remodelling of the local chromatin structure by SWI/SNF; displacing or 'sliding' nucleosomes and histone H1, which could subsequently enable the binding of secondary transcription factors and induce transcriptional activity of the target gene (Richard-Foy and Hager 1987; Becker et al. 1987; Piña et al. 1990; Archer et al. 1991; Reik et al. 1991; Rigaud et al. 1991; Bresnick et al. 1992; Ostlund Farrants et al. 1997). Importantly these studies used the nuclease enzymes DNase I and micrococcal nuclease (MNase) to assess chromatin accessibility by detecting the sensitivity of receptor binding sites and surrounding chromatin to nuclease digestion. Since then, coupling of these nuclease digestion techniques with the advancement of microarrays and

high-throughput sequencing has enabled DNase I hypersensitivity sequencing (DNase-seq) (John et al. 2011) and MNase sequencing (MNase-seq) (see review in Zentner and Henikoff 2012). This, in addition to the development of formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq) and chromatin immunoprecipitation (ChIP) combined with high-throughput sequencing (ChIP-seq) or microarrays (ChIP–chip), has allowed the study of accessible DNA regions, epigenetic marks and DNA–protein binding over the entire genome of a population of cells, for many different cell types for a wide number of steroid receptors in many different experimental conditions and contexts (see Furey 2012 and Zentner and Henikoff 2014 for further information on these and emerging genomic techniques). Indeed, not only have these techniques been instrumental in elucidating the principles of steroid receptor-mediated transcription, but they have revealed high levels of genomic organisation and enabled the identification of epigenetic marks and chromatin characteristics associated with active and repressed genes and regulatory domains (Heintzman et al. 2009; Thurman et al. 2012; Biddie 2011; George et al. 2011; Consortium et al. 2012; Zentner and Scacheri 2012). DNA methylation of CpG bases, nucleosome variants and histone modifications have been found to act in combination to dictate the chromatin architecture of a cell and define areas of DNA accessibility for protein binding. For example, DNAse accessibility and the histone marks of H3K27 acetylation and H3K9 acetylation are associated with enhancer and promoter regions that are undergoing gene expression, whilst the histone marks H3K27me3 and H3K9me3 are associated with repressed regions of chromatin (Consortium et al. 2012). These marks are often programmed during development and cellular differentiation to create highly tissue-specific chromatin landscapes of DNA accessibility, enabling one genome to produce many different cell-specific transcriptional programmes (Consortium et al. 2012; John et al. 2008, 2011; Natarajan et al. 2012). Indeed steroid receptors are found to bind thousands of genomic regions including promoter regions, as well as revealing large proportions of steroid receptor binding at distal enhancer regions, in highly cell- and context-specific binding patterns (Carroll et al. 2006; Kininis et al. 2007; Bolton et al. 2007; Reddy et al. 2009; John et al. 2011; Polman et al. 2013).

2.2.2 The Majority of Transcription Factor Binding Sites Are Preprogrammed to Be Accessible for Transcription Factor Binding

One of the most unexpected discoveries of genome-wide profiling has revealed that despite GR's characterisation as a pioneer protein at the MMTV array and other candidate genes (Richard-Foy and Hager 1987; Archer et al. 1991; Reik et al. 1991), the majority of GR binding in response to dexamethasone (95 % in AtT-20 cells) occurs at areas of accessible chromatin, termed 'preprogrammed' DNase I hypersensitive sites (John et al. 2008, 2011). GR only induced the de novo remodelling of chromatin at a

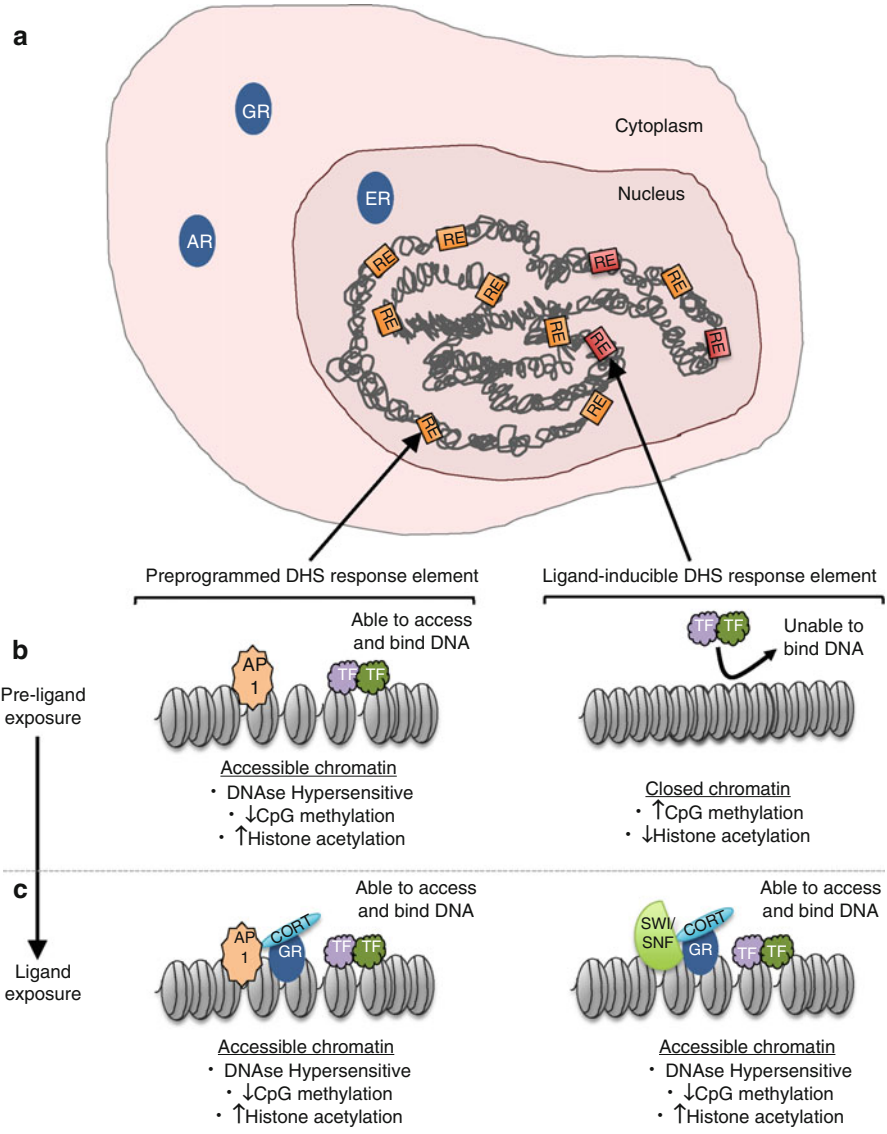
small proportion of binding sites (John et al. 2008, 2011; Biddie et al. 2011). Notably these patterns of DNase accessibility at transcription factor and GR binding sites are highly cell specific (Natarajan et al. 2012; Grøntved et al. 2013) with only 11.4 % of the GR binding sites found in pituitary cells also present in mammary cells (John et al. 2011). Numerous other transcription factors, including ER, PR and AR, have since been found to preferentially bind predetermined accessible regions of chromatin, in tissue- and context-specific manners, thus implying that predetermined chromatin accessibility is a generalised regulatory mechanism for determining transcription factor binding regions (He et al. 2010; Thurman et al. 2012; Ballaré et al. 2013; Miranda et al. 2013).

Strikingly genome-wide binding studies have identified that transcription factor binding sites are often clustered together. In the case of preprogrammed DNase I hypersensitive steroid receptor binding sites that exhibit open chromatin prior to steroid hormone exposure, other transcription factors that colocalise with the steroid receptor binding sites are thought to act as 'pioneer proteins' for steroid receptors and maintain open chromatin for hormone receptor binding prior to hormone exposure (see Fig. 2.1). For instance, the binding sites of ER in MCF7 breast cancer cells and AR in LNCaP prostate cancer cells have been found to predominantly associate with regions bound to FOXA1 (Carroll et al. 2005, 2006; Lupien et al. 2008), whilst in Att-20 cells 51 % of GR binding sites were colocalised with AP-1 binding sites (Biddie et al. 2011). Expression of an AP-1 dominant negative (A-Fos) reduced chromatin accessibility of preprogrammed GR binding sites that were localised with AP-1 regions, reducing GR binding and implying that AP-1 acts as a pioneer protein for GR (Biddie et al. 2011). Similarly knockdown of FOXA1 in MCF7 cells reduced ER DNA binding and decreases ER-mediated gene expression (Carroll et al. 2005).

Notably, however, the active chromatin epigenetic signatures H3K4me1 and H3K4me2 appear to precede FOXA1 binding at certain regions (Lupien et al. 2008), suggesting that stable (rather than dynamic) pre-existing chromatin modifications play an important role in regulating the accessibility of steroid receptor binding sites. These modifications are likely to result from processes specifying chromatin modifications and traits made during embryonic development and the differentiation of cell types (Polo et al. 2010; Kim et al. 2010). In addition DNA binding of a pioneer factor alone is not sufficient to induce ER binding. ER only interacted with approximately 20 % of the total FOXA1 binding sites, highlighting the presence of other regulatory mechanisms in determining the ER binding response (Lupien et al. 2008). DNA sequence and CpG methylation have also been implicated in determining transcription factor binding patterns influencing chromatin accessibility at enhancers and promoters. Strikingly, for GR binding, not only have preprogrammed DNase hypersensitive binding sites been found to contain a higher CpG density than surrounding regions, but they are also associated with decreased CpG methylation (Wiench et al. 2011). In fact tissue-specific preprogrammed GR enhancer binding sites tend to have a lower CpG enrichment compared to those that are shared across different cell types, whilst DNA binding sites that undergo de novo DNase accessibility upon GR binding are more likely to contain a low level of CpG enrichment and undergo demethylation upon GR activation (Wiench et al. 2011). Therefore, whilst the accessibility of a vast number of enhancer and promoter binding sites

appears to be preprogrammed, those that undergo de novo accessibility changes upon steroid hormone receptor activation seem to experience dynamic changes, not only in DNAse accessibility but also CpG hypomethylation, with methylation levels reducing to minimum levels within 10 min of dexamethasone exposure.

Whilst it is not yet known whether CpG density and hypomethylation is a cause or effect of preprogrammed DNase hypersensitive sites, siRNA-mediated depletion of DNA methyltransferase 1 (dnmt1) managed to decrease CpG methylation of several GR binding domains. However, although this resulted in a small change in



chromatin accessibility, it did not enable an increase in GR binding, suggesting that although demethylation and dnmt1 may mediate chromatin accessibility to some extent, CpG demethylation is not the only factor influencing the ability of GR to bind DNA (Wiench et al. 2011).

Recently, the importance of preprogrammed chromatin accessibility for GR binding sites has been demonstrated in vivo using mammalian tissue, where 82 % of GR binding sites occurred at preprogrammed accessible chromatin regions in the mouse liver (Grøntved et al. 2013). Notably of the genes that underwent transcriptional induction or repression (measured by RNA pol II ChIP-seq) 36 % of induced genes demonstrated a de novo increase in DNase hypersensitivity within 50 kb of the TSS (which appeared to be dependent on direct GR–DNA binding interactions), whilst 19 % of repressed genes decreased in accessibility at a proportion of loci (Grøntved et al. 2013). Chromatin remodelling and increased accessibility by GR therefore may play a more significant role in vivo compared to in vitro as in Att-20 pituitary cells 95 % of the GR binding sites were found to be accessible to DNase I prior to hormone addition. However, it must be noted that in these same AtT-20 cells, GR binding was associated with a 99 % increase in DNase I hypersensitivity upon hormone addition (John et al. 2011). Therefore, GR does still appear to modify chromatin structure and accessibility at most binding sites in a dynamic manner, although the degree of change depends on the region. This is supported by evidence from Burd et al. (2012) who used FAIRE-seq (Simon et al. 2012) to identify areas of low nucleosome occupancy across the genome. They found that hormone exposure was associated with a SWI/SNF-dependent increase in FAIRE signal (indicating a decrease in nucleosome occupancy) at most GR binding sites, even those that

Fig. 2.1 Nuclear receptor binding sites – structure of 'preprogrammed' and ligand-inducible DNAse hypersensitive response elements. (**a**) When activated by ligand binding, nuclear receptors are able to interact and bind directly to DNA at specific sequences termed response elements (REs). For instance, upon binding of corticosterone (cort), glucocorticoid receptors (GR) bind to specific glucocorticoid response elements (GREs), the vast majority of which have been found to be 'preprogrammed' to be hypersensitive to DNAse treatment (DHS) when tested prior to receptor activation (orange REs). However, GR can also bind some RE sequences that are located in inaccessible regions of chromatin that are not hypersensitive to DNAse digestion (red REs). (**b**) In the absence of ligand preprogrammed DHS, response elements are associated with an accessible chromatin conformation exhibiting low CpG methylation and high histone acetylation, which are induced/maintained by pioneer factors such as AP-1 or FOXA1. As other transcription factor response elements are often located close to nuclear receptor response elements, open chromatin in these regions can also be bound by other transcription factors (purple and green transcription factors (TFs)) prior to nuclear receptor activation. At ligand-inducible DHS response elements before ligand exposure, chromatin surrounding the RE is tightly compacted, exhibiting high CpG methylation and low histone acetylation; other transcription factors with binding sites in this region are prevented from binding to DNA. (**c**) Upon ligand exposure and nuclear receptor activation, nuclear receptors are able to bind response elements and regulate target genes at both preprogrammed and ligand-inducible DHS sites. At ligand-inducible DHS response elements, binding of GR results in an increase in chromatin accessibility and DNAse hypersensitivity, enabling itself and other transcription factors to bind to previously inaccessible regions of chromatin and regulate the expression of target genes

were already DNase 1 hypersensitive (Burd et al. 2012). Burd et al. suggest that most GR binding sites exist in a transitional state between an opened and closed chromatin conformation. This chromatin is open enough to be accessible to GR and for digestion by DNase I, but undergoes further remodelling upon GR binding (Burd et al. 2012). This is supported with evidence from genome-wide studies of PR binding which (unlike previous studies into GR binding) also performed MNase-seq (Ballaré et al. 2013). In this study even preprogrammed DNase I accessible PR binding sites exhibit a high nucleosome occupancy prior to hormone exposure, which subsequently underwent nucleosome depletion upon PR binding (Ballaré et al. 2013).

In summary, the binding of steroid receptors appears to be influenced by several overarching levels of regulation. Whilst steady-state, pre-existing epigenetic marks and chromatin landscapes can determine steroid receptor binding patterns via regulation of chromatin accessibility in a cell- and context-specific manner, further rapid dynamic changes to CpG modification and chromatin accessibility are induced at many receptor binding sites within minutes of hormone addition and are vital to transcriptional regulation. Indeed it must be stressed that although advances in DNAse-seq, MNase-seq, FAIRE-seq, ChIP–chip and ChIP-seq have been highly beneficial to our understanding of the binding site landscape, these techniques are unable to detect very fast temporal resolutions and are only able to distinguish between time points at a resolution of several minutes. Imaging studies in living cells however have discovered that protein–DNA binding interactions oscillate over timeframes of seconds.

2.3 Transcription Factor Interactions with DNA Are Highly Dynamic

Initial work on transcription factor DNA binding assumed transcription factors statically interacted with the DNA template to stably bind DNA over long periods of time. However, developments of fluorescently tagged receptors, including GR (GFP-GR), and photobleaching technologies, such as fluorescent recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), have revealed that the interactions between steroid receptors, coactivators, transcriptional machinery and the DNA template are highly dynamic. Using an array comprised of around 200 copies of the MMTV promoter, during constant hormone exposure GFP-GR was found to quickly dissociate from the DNA after the induction of an accessible chromatin state and then rapidly associated with the array again to facilitate or ‘assist’ binding of subsequent transcription factors, in a ‘hit-and-run’ mode of action (Mcnally et al. 2000; Fletcher et al. 2000; Nagaich et al. 2004). Notably the glucocorticoid ligand remains bound to the receptor during the fast cycling of GFP-GR on and off the DNA template (Meijsing et al. 2007). Other members of the nuclear receptor family including AR (Klokk et al. 2007), ER (Sharp et al. 2006) and PR (Rayasam et al. 2005) also exhibit rapid binding and displacement from the

DNA template. Indeed this behaviour is exhibited by other transcription factors such as NFκB (Bosisio et al. 2006) and numerous chromatin remodelling factors, coactivator proteins and RNA pol II (Becker et al. 2002; Nagaich and Hager 2004; Johnson et al. 2008). Although concern had been raised that these findings may not translate to endogenous regulatory sequences, Voss and colleagues (Voss et al. 2011) have recently investigated several endogenous regulatory regions of immortalised mouse mammary carcinoma cells (derived from 3617 cells). Using ChIP and a mutated ER protein (named ER pBox) in which the ER DBD was mutated to recognise GRE sequences, they found that coactivation of ER pBox and GR did not reduce GR binding at endogenous promoter regions. Not only was the binding of ER pBox and GR found to occur in a noncompetitive manner, but at regions that underwent de novo chromatin remodelling in response to glucocorticoid exposure, GR binding aided the subsequent binding of the ER pBox protein (Voss et al. 2011). This further supports the 'assisted loading' model of GR action implied earlier by the candidate gene studies of GR-mediated transcription from the 1980s and 1990s (Rigaud et al. 1991; Archer et al. 1992).

Strikingly although most receptors appear to have very rapid interactions with DNA, the time the transcription factor is associated with the DNA appears to affect the stability and efficiency of transcriptional induction (Stavreva et al. 2004; Lickwar et al. 2012). For instance, dexamethasone-activated GR has a slower exchange rate with the MMTV array (measured by FRAP) than corticosterone-activated GR, and induces transcription more robustly (Stavreva et al. 2004). The half-life of FRAP recovery for agonist-bound AR is also significantly longer than for antagonist-bound AR molecules, meaning that antagonists associate with the array for a shorter period of time (i.e. cycle more rapidly) in a manner that correlates with a decreased transcriptional activity of the receptor (Klokk et al. 2007). A recent genome-wide assessment of the transcription factor binding dynamics in yeast using a competition ChIP technique has shown that the longer residency times of the transcription factor interaction with DNA, the higher the transcriptional output (Lickwar et al. 2012). Therefore, the dynamics of this 'rapid fast-cycling' transcription factor binding to the DNA appears to be an important factor in determining the transcriptional output of the cell and may have the potential to change depending on the receptor structure induced by ligand binding.

2.4 Ultradian Oscillations of Steroid Receptor-Mediated Transcription

In addition to the fast-cycling protein–DNA interactions (timescale of seconds), longer-acting 'ultradian' oscillations of DNA–protein interactions, chromatin modification and transcriptional activity (timescale 45–90 min) have been identified during steroid receptor-mediated transcription. Elegant studies investigating the temporal dynamics of cofactor and chromatin modifications in human cells during ERα-mediated transcription of the cathepsin D promoter (Shang et al. 2000) and the pS2

(also known as trefoil factor 1; TFF) promoter revealed that constant oestradiol (E2) exposure results in the ordered and cyclical acetylation of histone H3 and H4, DNA methylation and the recruitment of coactivators (SRC proteins and HATs), corepressors (HDAC 1 and 7), SWI/SNF and RNA pol II in response to ER–DNA binding (Métivier et al. 2003, 2008; Kangaspeska et al. 2008). Indeed Métivier et al. assessed the temporal binding patterns of 30 different transcriptional proteins, using a kinetic ChIP experiment that assessed protein binding at the pS2 promoter, at 5 min intervals during a 3 hour oestradiol incubation, to identify an initial 'non-productive' cycle of ER binding that did not recruit RNA poll II, followed by approximately 40–45 min cycles of cyclical ER-induced transcription (Métivier et al. 2003). Further follow-up studies revealed that each transcriptionally productive cycle at the *pS2* promoter ended with cyclical 5-methyl CpG methylation and dnmt1 and dnmt3a/b occupancy, providing the first evidence of rapid reversible changes in CpG methylation during transcription, in a phenomenon that has also been replicated at several other ER-responsive promoters suggesting it may be a generalised mechanism of ER-mediated transcriptional oscillations (Kangaspeska et al. 2008; Métivier et al. 2008).

AR has also been found to undergo similar oscillations in DNA binding during constant testosterone exposure in LNCaP cells at the human prostate-specific androgen (PSA) and kallikrein-related peptidase 2 promoters, albeit with cycles 90 min in duration. Again, oscillations in histone H3 acetylation, CBP recruitment and RNA pol II recruitment were also observed following AR binding (Kang et al. 2002). Interestingly, however, treatment with a proteasome inhibitor was found to inhibit AR release from the promoter, abolishing the cyclical pattern of AR binding at the PSA promoter and resulting in constant AR occupancy of this regulatory region. In addition in the presence of a proteasome inhibitor, ER–DNA binding cycles at the pS2 promoter display an elongated periodicity of oscillations and fail to recruit RNA pol II (Reid et al. 2003). Degradation of ligand-bound AR and ER molecules by the proteasome is thus thought to play an essential role in mediating the oscillatory transcriptional profile during constant steroid exposure (Kang et al. 2002).

In contrast to the above examples of 'ultradian' oscillations of steroid receptor DNA binding and gene regulation that can occur during constant hormone stimulation, it is also important to note that transcriptional dynamics can also be directed by ligand availability. A prime example of this is the pulsatile activity of the glucocorticoid receptor, which has been proposed to play a key role in differentiating the body's response between stress and normal physiological glucocorticoid secretion (Fig. 2.2a).

2.4.1 Ultradian Glucocorticoid Secretion and Pulsatile Transcriptional Regulation

Notably the vast majority of studies into GR regulation of cofactor recruitment and chromatin structure and transcription have been performed using prolonged exposures to large doses of the synthetic ligand dexamethasone and focused solely on transcriptional induction, ignoring the rhythms of ligand exposure exhibited by

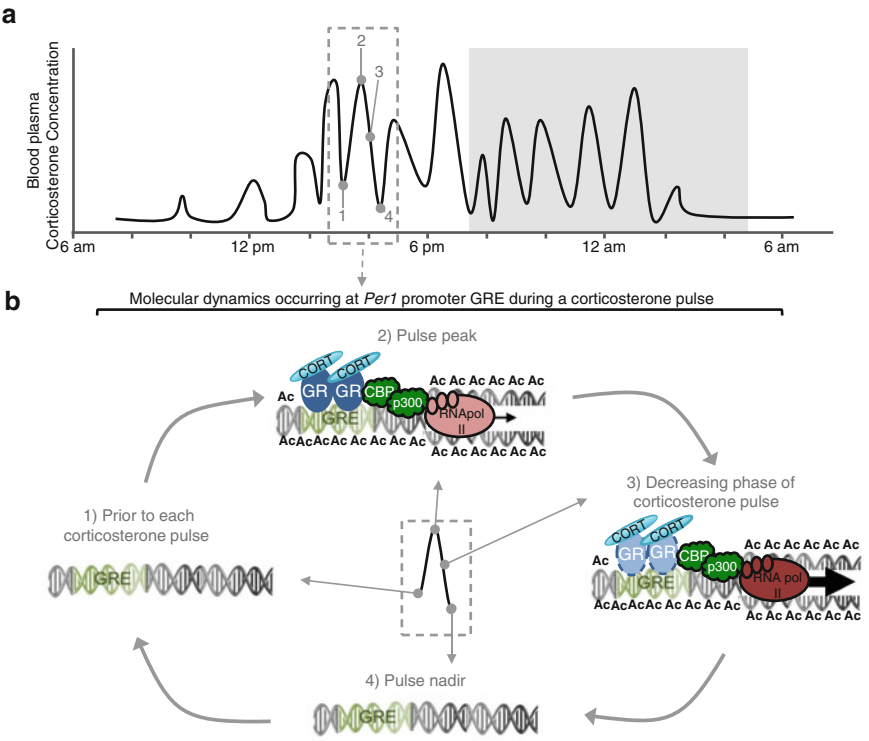


Fig. 2.2 Ultradian glucocorticoid exposure and the dynamics of GR binding, HAT recruitment and RNA pol II activity during a corticosterone pulse. (**a**) Schematic showing the pulsatile pattern of total corticosterone levels in an intact male Sprague-Dawley rat blood plasma, sampled at 10 min intervals (adapted from Lightman et al. 2008). Levels of plasma corticosterone rise during the sleeping phase of the rat during the early afternoon after which peaks are produced at approximately hourly intervals. Peak amplitude decreasing at the end of the waking phase of the rat, returning to basal levels at approximately 2 am. Shaded area represents lights off. Area in dashed box represents a typical corticosterone pulse, with numbers representing different phases of the molecular response to corticosterone. (**b**) Diagram showing the molecular response at the Per1 promoter during a single corticosterone pulse over a 60 min period from in vitro data (Conway-Campbell et al. 2011). Phase *1*: Prior to a rise in corticosterone levels, the Per1 promoter GRE exhibits basal levels of histone acetylation and DNAse accessibility, and no GR is bound to the GRE as it is unable to bind corticosterone at low corticosterone concentrations. Phase *2*: As corticosterone levels increase and reach their peak concentration, GR binds to corticosterone, becoming activated, binds to GRE sequences and is able to recruit the HATs CBP and P300 resulting in increased acetylation and accessibility of the promoter region, enabling RNA pol II recruitment and activation. Phase *3*: As corticosterone levels decrease following the peak of the corticosterone secretion, levels of ligand-bound GR decrease and binding GR decreases at the promoter region; however, levels of CBP, P300 and histone acetylation remain high and RNA pol II reaches its maximal occupancy and transcriptional activity. Phase *4*: By the time corticosterone levels return to their basal level (60 min after the rise of corticosterone levels), the Per1 GRE has returned to its original levels of acetylation, with minimal GR, HAT and RNA pol II binding, and thus can undergo a repetition of phases *1–4* when corticosterone concentrations rise with the next pulse

the endogenous glucocorticoid secretion in non-stressed healthy individuals. This is a critical oversight, as under basal non-stressful conditions, the secretion pattern of endogenous glucocorticoids (cortisol in humans, corticosterone in rodents) is produced from the adrenal glands in a highly dynamic pattern (Spiga et al. 2014). Unstressed, healthy mammals exhibit a well-characterised daily (circadian) rise in glucocorticoid secretion, controlled by the suprachiasmatic nucleus of the hypothalamus, that peaks prior to waking (early morning in humans, evening in rodents) and reaches a nadir by the rest period (evening in humans, morning in rodents; see Lightman and Conway-Campbell 2010). Furthermore, high-frequency blood sampling reveals that underlying this circadian cycle of glucocorticoid secretion lies a pulsatile ultradian glucocorticoid secretion pattern (Fig. 2.2a), which is evolutionarily conserved in all mammals studied, ranging from rodents to humans (Holaday et al. 1977; Mitsugi and Kimura 1985; Carnes et al. 1988; Veldhuis et al. 1989; Jasper and Engeland 1991; Cudd et al. 1995; Windle et al. 1998b). In rats these pulses occur at approximately hourly intervals (Jasper and Engeland 1991) during the active phase, whilst pulsatile cortisol secretion in humans occurs on average every 95–180 min (Follenius et al. 1987; Veldhuis et al. 1989), reflecting species-specific differences in HPA responsiveness and endogenous glucocorticoid metabolism. Notably this ultradian secretion pattern is intrinsically programmed within the HPA axis. The very nature of the feed-forward signals of CRH and ACTH secretion from the hypothalamus and pituitary to the adrenal gland, the short delay that it takes for the adrenal gland to synthesise corticosterone and the rapid non-linear feedback of glucocorticoids to the brain and pituitary which inhibits further ACTH secretion until glucocorticoid levels decrease create a circuit that induces a pulsatile pattern of glucocorticoid secretion by default (Walker et al. 2010, 2012). Importantly microdialysis studies in rats have demonstrated that biologically available levels of corticosterone pulsate not just in the periphery but also in the brain (Droste et al. 2008, 2009; Qian et al. 2012). Therefore, as GCs enhance/repress the transcription of many genes in many tissues, fluctuating GC levels affecting receptor binding could have profound effects on GC-regulated gene transcription.

2.4.2 Effects of Ultradian Glucocorticoid Secretion Pattern on Dynamics of Transcription

Notably glucocorticoids do not just activate GR, but can also bind and activate the mineralocorticoid receptor (MR). Both receptors are widely expressed throughout the body, and both can bind to the same GRE sequences in DNA to mediate transcription. However, despite their similarity, the two receptors exhibit major differences in their affinity for different glucocorticoid ligands (de Kloet et al. 1975). GR is activated specifically by glucocorticoids and has low binding affinity for endogenous corticosterone (c ~ 2.5–5 nM in the brain of Wister rats), whereas MR has a high corticosterone binding affinity (*Kd* ~ 0.5 nM), approximately 6–10 times higher

than that of GR (Reul and de Kloet 1985). The endogenous glucocorticoids corticosterone and cortisol therefore bind and activate MR at much lower concentrations than they do GR (Reul and de Kloet 1985; de Kloet and Reul 1987; Arriza et al. 1988). Therefore, it is believed that MR is fully occupied by ligand and transcriptionally active under a large number of circumstances even during low levels of corticosterone exposure. GR on the other hand, given its lower binding affinity for cortisol and corticosterone, has been proposed to be more responsive to the dynamic changes in glucocorticoid concentrations that result from endogenous glucocorticoid secretion (Krozowski and Funder 1983; Reul and de Kloet 1985; Arriza et al. 1988; Reul et al. 2000; Conway-Campbell et al. 2007).

Using an adrenalectomised rat model treated with exogenous intravenous (IV) bolus injections of corticosterone to mimic the ultradian glucocorticoid secretion pattern, the rapid temporal kinetics of GR and MR activation and DNA binding in response to a glucocorticoid pulses in vivo have been investigated (Conway-Campbell et al. 2007). Notably the pulsatile glucocorticoid exposure pattern induced distinct GR and MR nuclear translocation profiles, and MR nuclear translocation was induced at 10 min and persisted at a similar level for over 60 min; however, GR translocation more closely followed the corticosterone concentration over the time of each pulse, peaking at 15 min and returning to baseline by 60 min. Therefore, in line with evidence of GR and MR binding affinity, MR is proposed to undergo prolonged activation after glucocorticoid exposure and is likely to be continuously active even during troughs in ultradian corticosterone secretion. GR activation however closely follows fluctuations in corticosterone concentration (Kitchener et al. 2004; Conway-Campbell et al. 2007; Stavreva et al. 2009). Subsequent studies in vivo in the rat liver and Hippocampus revealed that sequential pulses of IV corticosterone administration at 60 min intervals resulted in distinct pulses of corticosterone exposure that induced hourly cycles of GR/DNA binding (measured by ChIP) and hnRNA (heteronuclear RNA/pre-mRNA) transcription of the *Period one* (*Per1*) clock gene (Fig. 2.2b). Stavreva et al. (2009) furthered the investigation of this phenomenon (which has been termed ‘gene pulsing’) to demonstrate cyclical GR/DNA binding at the *GluI* and *MT1* endogenous GRE in immortalised mouse cells, which were treated with 20 min pulses of 100 nM corticosterone at hourly intervals. These cells were also used to demonstrate pulsatile hnRNA production at the *Mt2*, *Tsc22d3*, *Tgm2* and *Suox* genes. Indeed subsequent analysis in AtT-20 cells that were stimulated with hourly pulses of corticosterone revealed highly dynamic changes in acetylation of the *Per1* promoter GRE 15 min after the application of each pulse (Fig. 2.2b) which coincided with the rapid recruitment of CBP/P300 to the promoter and a simultaneous increase in acetylated histone H4 levels (Conway-Campbell et al. 2011). Corticosterone was removed 15 min after the start of each pulse and by 30 min after the pulse application GR-GRE binding decreased. However, CBP/P300 and acetylated histone H4 levels remained elevated, and RNA pol II levels reached a peak occupancy at this time. Notably by 60 min after pulse application, occupancy of GR, CBP/P300, acetylated histone H4 and RNA pol II had returned to basal level, whilst the subsequent application of a further corticosterone pulse again resulted in the same cyclical occupancy patterns that were exhibited on application

of the first pulse (Conway-Campbell et al. 2011). Within the *Per1* gene, cyclical rises in RNA pol II occupancy correlate with pulses of hnRNA output (Conway-Campbell et al. 2011). In contrast, studies of transcription at the MMTV promoter during constant glucocorticoid exposure have demonstrated continuously induced levels of RNA pol II occupancy (above basal level) for at least 2 h even though transcription entered a lower refractory period after the initial induction (Becker et al. 2002). It therefore appears that constant glucocorticoid exposure does not result in intrinsically programmed cyclical cofactor and RNA pol II recruitment, in contrast to the behaviour of ER and AR. Instead ultradian oscillations in GR binding, cofactor occupancy and RNA pol II recruitment are directly programmed by the pattern of ligand exposure. This is an important distinction that may have implications for our understanding of glucocorticoid-associated diseases as dysregulation of glucocorticoid secretion by either treatment with synthetic therapeutic glucocorticoids or periods of chronic stress (both of which can result in prolonged GR activation far beyond the timeframe of ultradian GR activity) are associated with numerous negative health outcomes.

2.5 Disruption of Ultradian Glucocorticoid Secretion in Health and Disease

Interestingly live-cell imaging techniques have demonstrated that, compared to several synthetic glucocorticoid ligands, only the naturally occurring glucocorticoid ligands, corticosterone and hydrocortisone, were able to rapidly dissociate from GR, causing a cessation of GR/DNA binding within 15 min of hormone removal or 'washout' (Stavreva et al. 2009). Synthetic hormones such as dexamethasone caused more prolonged GR/DNA interactions, causing GR to remain continuously active even when dexamethasone was administered in hourly pulses (Stavreva et al. 2009). Strikingly Stavreva et al. demonstrated that cells undergoing pulsatile corticosterone exposure exhibited lower levels of nascent and mature transcripts, particularly for *MT2*, compared to cells constantly exposed to corticosterone. Furthermore, levels of *Fkbp5* protein were dramatically higher when stimulated by constant, rather than pulsatile corticosterone, implying that constant glucocorticoid exposure may lead to inappropriate protein expression (Stavreva et al. 2009). As the commonly used and frequently prescribed synthetic glucocorticoids (e.g. dexamethasone) also resulted in a prolonged GR activation, which has the potential to disrupt ultradian GR signalling, it has been proposed that continuous GR activation may account for the numerous adverse side effects that are associated with therapeutic glucocorticoid treatment (Lightman et al. 2008; Stavreva et al. 2009; Lightman and Conway-Campbell 2010). Thus, pulsatile glucocorticoid stimulation may have significant physiological importance in healthy HPA axis signalling, and its disruption may have significant clinical relevance (Young et al. 2004). This is supported by the fact that disruptions in glucocorticoid signalling

are implicated in numerous disorders such as cardiovascular disease (Walker 2007), osteoporosis (Seguro et al. 2013), metabolic syndrome (Tamashiro et al. 2011) and cognitive, memory and psychological disorders (Rome and Braceland 1951; Marin et al. 2011). High-frequency blood sampling of rats has demonstrated that HPA axis responsivity and circadian and ultradian activity is affected by many physiological and pathophysiological factors including lactation, weaning, aging, immunological activity and early life stress (Carnes et al. 1994; Atkinson and Waddell 1995; Cudd et al. 1995; Harbuz et al. 1999; Shanks et al. 2000; Windle et al. 2001, 2013). Genetics and epigenetics are also known to affect HPA axis secretion dynamics. The Fischer 344 rat strain, known to exhibit high anxiety, demonstrates bigger ultradian glucocorticoid pulses over a 24-h period, with less circadian variation than compared to a low-anxiety Lewis rat strain (Windle et al. 1998a). This disruption to the circadian secretion pattern (due to increased pulse amplitude during the circadian nadir) appears to be a common mechanism in HPA axis dysregulation and in high-stress states. Aging, for example, results in a flattened cortisol rhythm due to an increased level of glucocorticoids during the circadian nadir and a decreased level during the circadian peak (Carnes et al. 1993, 1994; Deuschle et al. 1997). In addition, compared to healthy control rats, rats with adjuvant-induced arthritis exhibited an increased rate of corticosterone secretion and a number of total pulses over a 24 h period, disrupting the circadian nadir (Harbuz et al. 1999).

HPA dysfunction has also been reported to be associated with disease in humans and is implied to pay a causative role in several pathologies including metabolic disorder and psychological disorders (Rome and Braceland 1951; Young et al. 2004; Lightman et al. 2008; Tamashiro et al. 2011; Kadmiel and Cidlowski 2013). Notably increases in pulse amplitude and/or the total amount of cortisol secreted have been associated with human disease and are observed in patients suffering from Cushing's syndrome (excessive glucocorticoid production; (Van Cauter and Refetoff 1985)) as well as in patients with alcoholism and hyperthyroidism that exhibit high cortisol levels (Iranmanesh and Veldhuis 1989; Johnson and Veldhuis 1990). Furthermore, evidence for a pathological role of disrupted glucocorticoid signalling comes from the adverse effects and elevated mortality associated with elevated glucocorticoid secretion in Cushing's syndrome (Etxabe and Vazquez 1994; Dekkers et al. 2013). Strikingly patients undergoing glucocorticoid therapy experience similar detrimental effects and increased mortality rates to those patients with Cushing's syndrome, suggesting that maladaptive physiological states may arise from altered GR activity (Clark et al. 1952; Wolkowitz et al. 1997; Hempen et al. 2002; Bergthorsdottir et al. 2006; Fardet et al. 2007; Bensing et al. 2008; Ross and Cetas 2012). Disruption of pulsatile glucocorticoid signalling, either by stress, disease or therapeutic glucocorticoid exposure, has therefore been proposed as a potential mechanism underlying the common adverse effects that are associated with these physiological situations by evoking an increase in the duration of GR receptor binding (Young et al. 2004; Lightman et al. 2008; de Kloet and Sarabdjitsingh 2008; Lightman and Conway-Campbell 2010).

2.6 Purpose of Dynamic Transcription Cycles in Normal Physiological Circumstances

Just as ultradian fluctuations in blood plasma corticosterone underlie the circadian rise in glucocorticoid secretion, it is hypothesised that the pulsatile fluctuations in cofactor recruitment and chromatin structure induced by ultradian GR activity may support circadian changes in chromatin structure that have been observed at the *Per1* gene (Naruse et al. 2004) and potentially other regions. The fast-cycling stochastic interactions of steroid receptors with DNA (Mcnally et al. 2000; Fletcher et al. 2002; Stavreva et al. 2004; Nagaich et al. 2004) are in addition likely to underlie both of the ultradian cycles in steroid receptor activity and chromatin modification elicited by GR, ER and AR (Fig. 2.3). This proposal of an interacting system of multiple overlying levels of oscillatory transcriptional activity is in concordance with evidence from transcriptional studies in yeast, which suggest that fast-cycling transcription factor interactions be responsible for engaging transcriptional initiation, whilst slow overlying cycles of activation are required for mRNA production (Karpova et al. 2008). Thus, steroid receptor-regulated transcriptional regulation appears to constitute a balance of overlying rhythms in receptor binding, chromatin modification and transcriptional activity.

Interestingly several studies have hypothesised about the importance of cyclical transcriptional activity and cofactor recruitment suggesting that oscillations may be required in order to 'reset' chromatin structure for subsequent cycles of transcription (Wang et al. 2009) and may maintain an efficient transcriptional

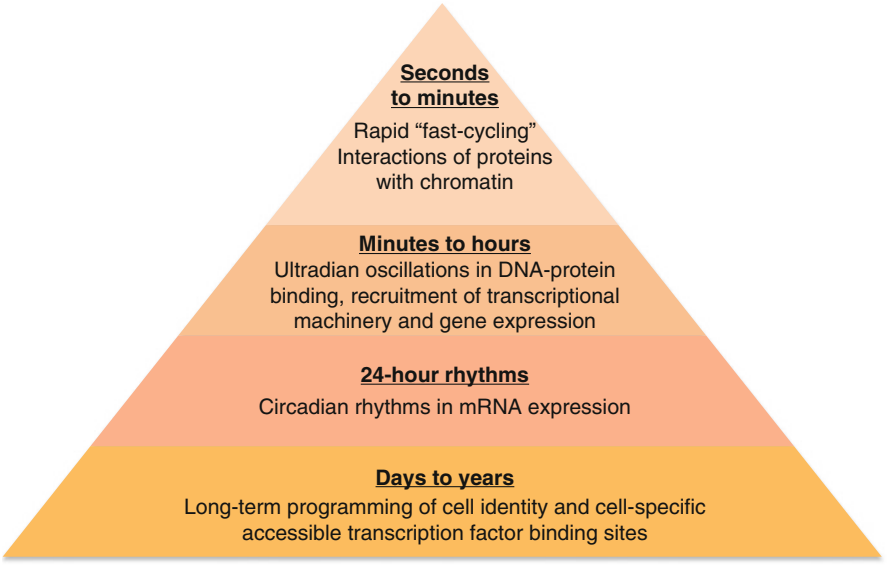


Fig. 2.3 Schematic illustrating the different timeframes that can underlie a transcriptional response. Durations of different timeframes are at the *top* of each line in *bold* and *underline* and a brief description of the molecular mechanisms that are relevant to each timeframe

system. It is possible that ultradian cycles of transcriptional molecules at promoters may enable cells to avoid refractory periods of low transcriptional output, which have been suggested to occur during constant glucocorticoid exposure at regions such as the MMTV promoter and at regulatory regions of several ER-regulated genes (Chen et al. 1999; Becker et al. 2002). Notably several studies have reported that there are limited and transcriptionally restricting concentrations of coactivator proteins in cells (Kamei et al. 1996; Sheppard et al. 1998). The overexpression of factors such as CBP and P300 is able to induce and enhance transcriptional responses (Smith et al. 1996). This has led to the concept of 'squelching' where activation of a transcription factor, such as GR, is proposed to indirectly downregulate the transcription of genes in other signalling pathways, by sequestering vital cofactors at GR-regulated genes (Kamei et al. 1996; Smith et al. 1996; Sheppard et al. 1998; Fonte et al. 2007). Given the highly dynamic temporal changes in cofactor recruitment provoked by ultradian transcriptional cycles, it is possible that prolonged periods of GR activation (e.g. during chronic stress, HPA axis hyperactivity or synthetic glucocorticoid administration) will induce extended periods of cofactor recruitment at GR-regulated genes. This may consequently result in the squelching of cofactors from other important signalling systems, disrupting their transcriptional regulation. Therefore, aberrant GR activity may not only dysregulate the transcriptional network of glucocorticoid target genes, but it could also effectively downregulate and disrupt the transcription of other genes that require the same cofactors.

In summary chromatin modification and cofactor recruitment in response to steroid receptor activation are highly dynamic, in contrast to the static model of transcription usually described during experiments involving prolonged ligand exposure and focusing solely on the induction of transcriptional regulation.

2.7 Conclusions

In summary, over the last 15 years, advances in molecular biology and imaging technologies have made it increasingly apparent that transcription factor DNA binding, cofactor recruitment, chromatin modifications and transcription are far more dynamic than previously thought. Instead of a static transcriptional model, gene regulation is controlled by numerous dynamic processes that occur on timescales with many different orders of magnitude. Although we have made vast leaps in characterising the dynamics of these transcriptional systems in recent years, it is highly likely that future advances in technologies will enable us to understand these processes further. Notably in the case of the ligand-driven pulsatile GR activation, understanding the ultradian dynamics underlying the glucocorticoid-mediated transcriptional programme may enable us to understand the disease process that can occur during prolonged glucocorticoid exposure such as during chronic stress or therapeutic glucocorticoid administration.

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Chapter 3
Nuclear Receptor Coactivators

Ioannis Zalachoras and Onno C. Meijer

Abstract The effects that steroid hormones exert on gene expression via their nuclear receptors (NRs) must be tightly regulated, in particular because of their pleiotropic effects in many tissues. To that end, regulation of receptor activity takes place at multiple levels, which include ligand availability, epigenetic modifications of chromatin around tissue-specific target genes, expression levels of the receptor, and the presence or absence of other NRs in the same cell. One of the levels of transcriptional control is that of the NR coregulators, proteins that can interact with NRs and modulate their function. Coregulators can interact with multiple NRs and NRs can interact with multiple coregulators. As a consequence, coregulator expression in certain cell types may play the roles of hubs and bottleneck that offers gene target, cell type, or context specificity. Below we offer an overview of NR coregulator function, highlighting the best-described coregulators in the brain, as well as possibilities for the manipulation of NR–coregulator interactions for therapeutic or experimental purposes.

Keywords Sex steroids • Glucocorticoids • Gene transcription • Selective modulators • Brain

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3.1 Introduction

Steroid hormones exert their effects in both the brain and the periphery, orchestrating a wide range of behavioral and physiological responses. Given the nature of neuroendocrine regulation, steroids not only act as final signaling molecules of neuroendocrine axes but they also shape the activity of these axes via direct negative feedback actions and more complex indirect feedback on the brain. Their effects are mediated by their respective nuclear receptors (NRs). NRs act in large measure as transcription factors that modulate gene expression and chromatin structure. They show a wide distribution pattern in peripheral target organs and different cell types in brain and pituitary (Gofflot et al. 2007). Given the wide range of possible steroid actions and the broad expression pattern of their receptors, it is important that their effects are regulated at various levels. Such regulation can take place at the level of the ligand availability, type and local concentration (Awasthi and Simons Jr 2012; Yang and Fuller 2012), the expression levels and posttranslational modifications of the receptor (Noguchi et al. 2010; Nicolaides et al. 2010), interactions with molecular chaperones in the cytoplasm (Hartmann et al. 2012; Touma et al. 2011), dimerization and translocation to the nucleus (Fitzsimons et al. 2008), the presence or affinity of multiple receptor types for the same ligand in the same cell (de Kloet et al. 2005), the presence and activity of kinases such as SGK-1 (Anacker et al. 2013), and, once the receptor is in the nucleus, the chromatin landscape and many interactions with proteins that interact with or compete for nuclear receptors (de Kloet et al. 2009). The latter can be divided into other – non-receptor – transcription factors and nuclear receptor coregulators.

3.2 Nuclear Receptors

All nuclear receptors consist of functional domains that can be directly coupled to their function as transcription factors and indirect chromatin modifiers. The relationship between the structure and the function of the nuclear receptors has been extensively studied and described (Mittelstadt and Ashwell 2003; Giguere et al. 1986). In short, the NR proteins are composed of three critical modular domains: a poorly conserved N-terminal domain that harbors the hormone-independent activation function 1 (AF1), a central DNA-binding domain (DBD) that shows extensive homology between related NR family members, and a C-terminal ligand-binding domain (LBD) that also forms the hormone-dependent activation function 2 (AF2) domain that is activated allosterically upon ligand binding (Fig. 3.1) (Mittelstadt and Ashwell 2003; Danielian et al. 1992; Giguere et al. 1986; Stanišić et al. 2010).

In the absence of ligand, NRs are bound to chaperone protein complexes in the cytoplasm, such as FKBP5 and HSP90 (Menke et al. 2013; Klengel et al. 2013; Picard et al. 1990). Upon ligand binding, a conformational change takes place that leads to the dimerization of the nuclear receptor and its translocation to the nucleus. There, the receptor binds to the DNA, either directly via its DBD or indirectly via

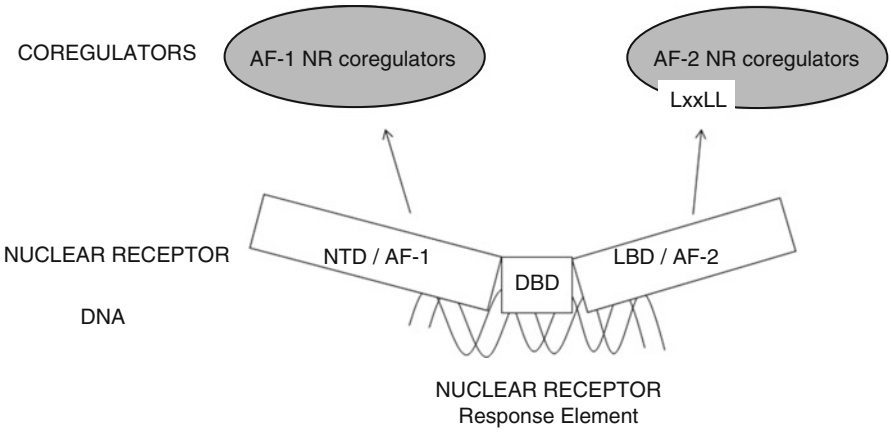

Fig. 3.1 The domain structure of nuclear receptors and their modes of coregulator interactions. The central DNA-binding domain (DBD) is flanked by the N-terminal domain (NTD) and the ligand-binding domain (LBD). The LBD has a well-conserved structure consisting of 12 alpha helices. These form activation function 2 (AF2) that contacts AF2 NR coregulators via their LxxLL motifs, or "NR-boxes." AF2 NR coregulators are shared between many NRs, and interactions can readily be identified using in vitro protein assays, such as MARCoNI. AF1 lies in the NTD – it is intrinsically unstructured, and in general, the AF1 NR coregulators are more specific to a particular receptor and more difficult to identify

interaction with other transcription factors. In the direct DNA-binding mode, molecular interactions with a host of transcriptionally active proteins may take place via the AF1 and AF2 domains. Direct DNA binding occurs at nuclear receptor responsive elements (NREs), specific nucleotide sequences for each NR linked to activation or repression of specific genes. There may be hundreds of thousands of NRE-like sequences in mammalian genomes (Datson et al. 2011), but chromatin structure and the demand of associated binding partners limit actual binding to only a couple of thousand detectable binding loci per cell type. However, these show a very substantial cell specificity (John et al. 2011), and even strong evolutionary conservation of response elements does not automatically imply responsiveness in a particular tissue or cell type (Datson et al. 2011). The receptors are thought to mainly form homodimers, act as monomers in conjunction with other non-receptor or transcription factors, or heterodimerize with other steroid receptors (Pearce 1994; Chen et al. 1997; Presman et al. 2014; Trapp et al. 1994). Of note, although initial promoter-directed study revealed a substantial number of response elements within hundreds of base pairs of transcription start sites, genome-wide approaches have revealed that NREs can be localized many kilobases away from the genes they regulate and may act *in cis* over very long ranges.

Other transcription factors can interact with NRs during DNA binding. Some of the identified transcription factors will bring the receptors to the DNA by way of "tethering" mechanisms, like those involved in classic transrepression in the immune system (De Bosscher et al. 2008). There are also those transcription factors that bind in the vicinity (within hundreds of base pairs) of the steroid receptors and

are in some way involved in modulating their function, also by chromatin and DNA modifications (Biddie et al. 2011).

3.3 Coregulators' Mode of Action

Nuclear receptor coregulators are proteins that interact with NRs but not the DNA (they are not transcription factors) (Zalachoras et al. 2013c). They have three main functions: (i) they can recruit other transcriptionally active proteins, (ii) they have histone acetyltransferase or methyltransferase activity and/or they can recruit histone acetyl- or methyltransferase, and (iii) they stabilize the transcriptional machinery (Tetel et al. 2009). In direct DNA-binding mode, the rate of transcriptional stimulation at a given ligand concentration is thought to be limited by any of the transcriptional coregulators of the receptors, in a gene-specific manner (Ong et al. 2010). In a general sense, the coregulator repertoire that is assembled at a particular locus on the DNA determines the magnitude as well as the nature of the transcriptional response.

The activities of the AF1 and AF2 output domains of NRs by definition depend on interactions with coregulators (Fig. 3.1). AF1 coregulators are difficult to predict and often relatively unique for particular NRs, based on the low degree of homology between the different receptors and the intrinsic unordered nature of the domain. AF2 domains are much more structured and conserved, and their coregulators share structural domains called NR-boxes. These contain specific motifs containing the amino acid sequence LXXLL. The AF2–NR-box interactions have revealed detailed structural information, which can be coupled to the conformational change of the receptor after binding to agonists or antagonists (Huang et al. 2010). In addition, the interactions may be screened for in vitro based on binding of the (recombinant) receptor protein to NR-box-containing peptide fragments from many different coregulators (Zalachoras et al. 2013a).

The coregulators that are recruited after hormone binding tend to be coactivators rather than corepressors. In this respect the classical steroid receptors differ from other classes of nuclear receptors, which may be DNA-bound in absence of ligand, but transcriptionally inactive based on corepressor binding. Interestingly, some steroid receptor antagonists, such as the mixed AR/GR/PR antagonist RU486/mifepristone, induce recruitment of corepressors rather than coactivators (Zhang et al. 1998). As discussed later in this chapter, this screening opens the possibility to find new steroid receptor ligands with intermediate coregulator recruitment profiles which consequently combine agonistic and antagonistic properties – so-called selective receptor modulators (Zalachoras et al. 2013b).

Cell-type-specific chromatin organization and coregulator repertoire (Meijer et al. 2000) (www.nursa.org) interact as the recruitment of coregulators by nuclear receptors may take place in a cell-type- and locus-specific manner (Trousson et al. 2007). Coactivators tend to either be direct modifiers of histones via acetyltransferase or methyltransferase activity or recruit other coactivators that subsequently change histone posttranslational modifications, such as CREB-binding protein (CBP) and many

others (Won Jeong et al. 2012). The ensuing histone marks may act as epigenetic determinants of cell fate of future cell behavior. This model indicates that coregulators do not act in isolation but in protein complexes that may involve transcription factors, coregulator–coregulator interactions, and RNA molecules (Tetel et al. 2009).

Steroid hormone treatment may also lead to epigenetic changes at the level of CpG methylation on the DNA (Auger et al. 2011; Yu et al. 2013; Zhao et al. 2010; Sharma et al. 2013). There is however not much evidence for direct recruitment of DNA methyltransferases by NR coregulators, although this would constitute a mechanism for gene-specific regulation of CpG islands. On the other hand, DNA methylation at specific loci may determine which coregulators are recruited by specific nuclear receptors (Ceschin et al. 2011).

With tissue- and cell-specific expression patterns, as well as their promiscuity (coregulators can often interact with multiple NRs, mostly based on common interactions with the relatively homologous LBD/AF2 domains of the receptors), coregulators can create an additional level of regulation that drives the pleiotropic effects of NRs toward cell-specific transcriptional changes, as well as to NR-specific expression programs when competition for coregulators occurs. Moreover, given their potential to induce chromatin modifications, coregulators may be the link between NR function and the appropriate epigenetic changes in response to stimuli (Hunter 2012). Below, we describe examples of interactions between NRs and coregulators that highlight the importance of coregulators for the steroid receptor family members with focus on brain function.

Estimates of the number of nuclear receptor coregulators are as high as >350 different proteins (Stanisić et al. 2010), but the importance of most of these for individual steroid receptors is unknown and not every coregulator interacts with every receptor type. It is also clear that there is a pronounced differential distribution of different coregulators per brain region (www.brain-map.org). Neuroendocrine relevance of individual coregulators discussed below, based on interactions found in cell lines or other organ systems, clearly depends on actual coexpression with steroid receptors in the brain and/or pituitary.

3.3.1 Sex Steroid Receptor Coregulators

AR plays important roles in the brain, most pronounced in regulating male sexual behavior. However, given therapeutic urgency, coregulator function in AR action has been extensively studied in relation to the development and progression of prostate cancer. For example, E6-associated protein (E6-AP) is a coregulator that interacts with AR during the development of the prostate gland, and it plays an important role in development of the brain, but it is not clear whether it is important for AR function in the adult brain.

In neuroendocrine setting, steroid receptor coactivator-1 (SRC-1) is the best-characterized AR coregulator (Feng and O'Malley 2014), and it has been shown to be necessary for regulation of the androgen-induced behavior and plasticity in Japanese quail (Charlier et al. 2006a). Blockade of SRC-1 expression in the brain

led to abrogation of testosterone-dependent sexual behaviors, as well as the testosterone-dependent growth of the preoptic medial nucleus, an area of the quail brain involved in sexual behavior (Charlier et al. 2006b). Interestingly, both AR and SRC-1 expression are regulated by photoperiod and testosterone treatment, indicating the significance of parallel regulation of these two components for signaling (Charlier et al. 2006a).

SRC-1 was the first of the classical coregulators to be described (Oñate et al. 1995) and belongs to the so-called p160 family. Other members such as SRC-2 and SRC-3 can also be involved in AR signaling, although their involvement has not been studied as extensively in the brain. Nevertheless, they may show some redundancy with SRC-1, as SRC-2 overexpression is a known compensatory mechanism in the absence of SRC-1 (Apostolakis et al. 2002), or offer differential regulation in certain brain areas (i.e., SRC-3 shows a very distinct pattern in the brain with high expression only in the hippocampus).

Estrogen receptors are expressed in two subtypes ERα and ERβ which are coded for by two different genes. The effects of the interactions between ER and coregulators have been broadly studied in relation to breast cancer, sexual behavior, and cognitive function. In the same context epigenetic regulation of the expression of ER-dependent genes has been shown to be relevant as well, in rather complex cascades of events. For instance, estradiol can control the expression of enhancer of zeste homolog 2 (EZH2), a methyltransferase specific for lysine 27 of histone 3, overexpressed in breast cancer, together with mixed lineage leukemia (a coregulator) and CBP/p300 (Bhan et al. 2014).

Next to many studies in relation to sexual function and differentiation, considerable work has been done on the effects of estrogens on stress responses and susceptibility (Calmarza-Font et al. 2012; Shansky and Lipps 2013). The fact that early life treatment with estrogen results in altered later life stress responses indicates that epigenetic mechanisms may be at play (Panagiotidou et al. 2014), something that is backed up by observation after manipulation of downstream DNA methylation factor expression (Wang et al. 2013b). The exact effects of estrogens (e.g., anxiolytic or anxiogenic) differ, depending on the age of treatment, the sex of the animals, and, more importantly, treatment with other steroid hormones or the expression levels of other steroid receptors.

In relation to specific coregulators that interact with ER, again most data collected indicate the involvement of members of the p160 family. They are largely coexpressed with ER in the rodent brain, interact with ER (Yore et al. 2010), and often follow the seasonal or age-dependent expression patterns of ER (Tetel et al. 2007; Tognoni et al. 2011). Moreover, SRC-1 expression varies in the hypothalamus of cycling female rats with a nadir during diestrus and a zenith during proestrus and estrus (Charlier et al. 2010). Furthermore, in the brains of aged female mice, SRC-1 has lower expression, indicating a reduction in hormone sensitivity. Experimental deletion of SRC-1 and SRC-2 expression in the brain resulted in loss of sensitivity to estrogens leading to aberrant hormone-induced sexual behavior in female rats, which was not recovered even after high doses of estradiol (Apostolakis et al. 2002), even if other studies reported similar but much less striking effects (Molenda et al. 2002). Also in male Japanese quail, intracerebroventricular injections of antisense

oligonucleotides targeting SRC-1 blocked the estrogen-dependent sexual behaviors (Charlier et al. 2006b).

Another interesting ER coregulator is ribosomal protein L7 (RPL7). This protein is a selective coactivator of the ER involved in mRNA translation, and in avian species, it is highly expressed in the brain and particularly in the regions involved in song control such as HVC, RA, and area X (Duncan et al. 2009). Sexual dimorphism in its expression in the brain has been reported. It is believed that its upregulation at a certain age may play an additional role in the sexual differentiation of the avian brain in response to estrogens (Duncan and Carruth 2011). Interestingly, in vivo knockdown of RPL7 in the zebra finch brain resulted in altered morphology in song control regions, without however any differences in song learning and singing behavior, possibly related to other coregulators that can take over RPL7's function in its absence.

Recently, data collected postmortem from the brains of patients with autism spectrum disorders have shown that ERβ, together with SRC-1, CBP, and P/CAF, has reduced expression in the medial frontal gyrus compared to controls (Crider et al. 2014). Combined with data showing the effects of the AR and ER together with coregulator NCOA5 on the retinoic acid receptor-related orphan receptor A (RORA) promoter may suggest that sex hormones may be relevant for autism spectrum disorders, as well as for the sex bias in the development of such disorders (Sarachana and Hu 2013a, b). Models of RORA insufficiency show behavioral patterns similar to autism spectrum disorders such as spatial learning deficits and reduced object exploration (Sarachana et al. 2011).

3.3.2 GR and MR Coregulators

Among other functions, GR and MR orchestrate the expression of responses to stressors, which involves the coordination of multiple systems in the brain and the periphery (Myers et al. 2013; Herman 2013; Rodrigues et al. 2009; de Kloet et al. 2005). The HPA axis plays a central role in the regulation of stress responses via control of glucocorticoid hormone levels. Glucocorticoids, in turn, exert a wide range of effects, including effects on memory, behavior, and metabolism, that are mediated by their receptors MR and GR. Importantly, glucocorticoids can block the expression and release of CRH in the PVN and *POMC*/ACTH in the pituitary, thus creating a negative feedback loop (Kovács 2013; Laryea et al. 2013). Of interest, there is at least one coregulator – the SRC-1 splice variant SRC-1a – that is enriched in the hypothalamus and pituitary and may mediate transcriptional effects that are part of negative feedback actions (Meijer et al. 2000). Many coregulators are shared between MR and GR, even if some functional differences exist (Meijer et al. 2005). Specific coregulators likely act via the poorly conserved AF1. Recently, Gemin4 has been shown to function as an MR coregulator, as well as 11–19 lysine-rich leukemia (ELL) (Yang et al. 2015; Yang and Young 2009; Pascual-Le Tallec et al. 2005). Within the brain, MR- and GR-specific coregulator pathways are basically unknown.

In the multiple GR- and MR-dependent neuromodulatory pathways, some of which result in epigenetic changes (Hunter et al. 2014), several coregulators take part. Many studies have been conducted on the coregulators of GR and to a lesser extent MR in relation to brain function, particularly for learning and memory and stress responses. Apart from members of the p160 family, one dominant example is the coregulators of CREB CBP/p300, which via p160 interaction are secondary coregulators of steroid receptors. Other examples are p300/CBP-associated factor (pCAF), members of the CREB-regulated transcription coactivator (CRTC) family, and the coregulators of steroid hormone receptors RIP-140 and Ube3a (Barrett et al. 2011; Malvaez et al. 2011; Oliveira et al. 2007; Jeanneteau et al. 2012; Ch'ng et al. 2012; Augereau et al. 2006; Duclot et al. 2010, 2012; Maurice et al. 2007; Godavarthi et al. 2012; Mardirossian et al. 2009; Wallace et al. 2012; Weeber et al. 2003; Engel and Yamamoto 2011). Not surprisingly, mutations or deletions of these coregulators often result in impairments in learning and memory, decreased neuronal plasticity, inappropriate regulation of stress responses, or abnormal brain morphology (Zalachoras et al. 2013c).

SRC-1 and the other p160 family members are arguably the best-characterized GR coregulators. SRC-1 has been shown to be crucial for GR-dependent regulation of CRH expression in the PVN and the central nucleus of the amygdala (Lachize et al. 2009). SRC-1 is expressed in two splice variants, SRC-1a and SRC-1e, which have differential distribution in the brain and opposite activities on the *crh* promoter (Fig. 3.2) (van der Laan et al. 2008; Meijer et al. 2000, 2005). SRC-1a downregulates *crh* expression and is highly expressed in the PVN, while SRC-1e lacks repressive activity and shows high expression in the CeA. In SRC-1 KO animals, *crh* expression in the PVN and the CeA is largely resistant to regulation by glucocorticoids, as well as POMC expression in the anterior pituitary, while *crh* expression in the CeA is

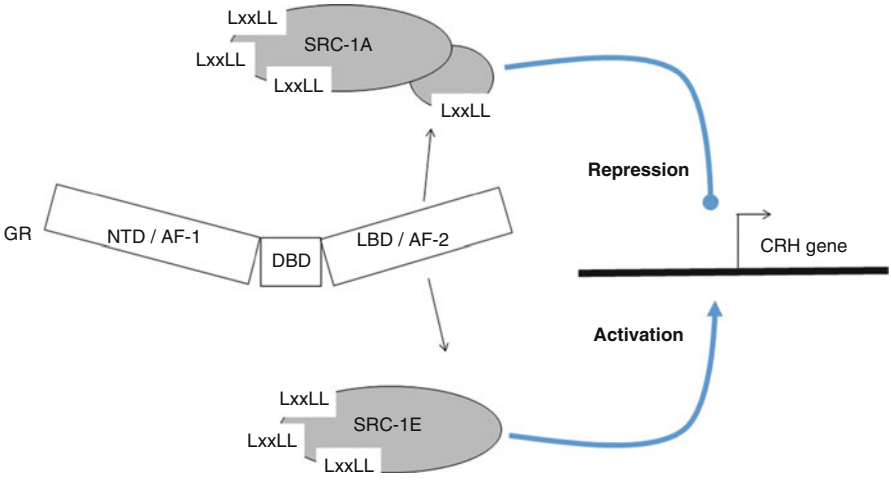

Fig. 3.2 Proposed model of SRC-1 splice variant differential action. SRC-1a has a longer C-terminal domain that contains an additional LxxLL NR-box, as well as a repressor function. Its recruitment by GR can lead to repression of the *CRH* gene, whereas SRC-1e may act as a stimulatory coactivator

decreased (Lachize et al. 2009; Winnay et al. 2006). The HPA activity and behavioral responses to stress are close to normal in these animals, despite the transcriptional phenotype. This can be partly attributed to the compensatory developmental upregulation of SRC-2 expression in the absence of SRC-1 (Nishihara et al. 2003), and definitive conclusions await transient or inducible genetic experiments. Despite the extensive study of the SRC-1 splice variant function in vitro, only recently have there been attempts to study their function in vivo utilizing exon skipping methods to shift the expression ratio of SRC-1 and SRC-1e in the mouse brain (Zalachoras et al. 2013a). On the other hand, apart from the compensatory effects of SRC-2 in the absence of SRC-1, deletion of SRC-2 results in impaired adrenocortical output at the level of the adrenal, thus increasing the HPA axis in response to stress. Interestingly, the deletion of any of SRC-1, SRC-2, and SRC-3 was shown to have effects on anxiety behavior, which were often sex-dependent (Stashi et al. 2013).

CREB-binding protein (CBP) is a potential brain GR (Conway-Campbell et al. 2011) and MR coregulator (Kitagawa et al. 2002). CBP is a HAT that can be recruited by SRC-1 and therefore likely is a secondary coregulator to all steroid receptors. CBP and its homolog p300 (Barrett et al. 2011) are also downstream transcriptional modulators of CREB and AP1. Since gene transcription is essential for many processes such as learning and memory and stress responses, as well as a key mode of action of steroid receptors, the broad involvement of CBP/p300 in such processes is not surprising (Maurice et al. 2007; Malvaez et al. 2011). GR effects on learning and memory may be to some extent CBP/p300 dependent (Roozendaal et al. 2010) either via LxxLL-dependent GR-CBP/p300 direct interactions or via recruitment by SRC-1. CBP/p300 has different LxxLL interaction domains for GR, one KIX domain for interaction with CREB, and one SRC-1 interaction domain; thus, combinatorial binding may be possible (Wang et al. 2013a; Waters et al. 2006; Chan and La Thangue 2001). Thus, a model has been proposed in which glucocorticoids can functionally interact with CBP and alter gene expression both by direct binding and promoter transactivation and by histone modifications (Roozendaal et al. 2010). Lack of CBP results in decreased histone methylation, together with impairments in long- and short-term memory (Barrett et al. 2011; Chen et al. 2010), while similar results have been observed after deletion of p300 (Oliveira et al. 2007, 2011). Interestingly, in the absence of CBP, p300 is not always upregulated and cannot take over all CBP-dependent functions, thus indicating a certain degree of non-redundancy of the functions of the two proteins (Barrett et al. 2011). Thus, CBP and p300 have significant involvement in learning and memory, and despite their homology, they have possibly non-redundant roles in these processes, although their roles may also be brain region dependent (Marek et al. 2011). The functional "integrator" CBP may well be one of the direct substrates of the close cross talk between GR and CREB pathways in neuronal plasticity.

CREB-regulated transcription coactivators (CRTCs) are primarily known as transcriptional coregulators of CREB. Upon cAMP and calcium exposure, they are dephosphorylated and translocate into the nucleus where they can interact with CREB over relevant promoters controlling the function of NRs (Liu et al. 2010, 2011, 2012; Altarejos and Montminy 2011). Jeanneteau et al. studied how BDNF, GR, and CREB

regulate *crh* expression (Jeanneteau et al. 2012). It had been known that BDNF can upregulate *crh* expression in the PVN (Givalois et al. 2004), whereas GR activation (e.g., after treatment with glucocorticoids) represses *crh* expression in the PVN (Makino et al. 1994). Jeanneteau et al. showed with a combination of loss- and gain-of-function techniques that there is a cross talk between GR and BDNF and its receptor TrkB through interactions with CREB, and mediation of the CRTC2 may activate the *crh* promoter while glucocorticoids through the GR may target phosphorylation and nuclear localization of CRTC2 and repress the *crh* promoter (Jeanneteau et al. 2012).

Another HAT involved in learning and memory that interacts both directly and indirectly (via p160 family members) with GR is p300/CBP-associated factor (PCAF) (He et al. 2002; Szapary et al. 2008; Blanco et al. 1998; Li et al. 2003), which can also acetylate other transcriptional regulators (Pérez-Luna et al. 2012). It has been found to be upregulated together with increased histone acetylation in the rat hippocampus during memory consolidation (Bousiges et al. 2010). Lack of PCAF resulted in impaired memory function, exaggerated stress responses, anatomical differences in their hippocampus, and decreased synaptic plasticity (Maurice et al. 2007), while its blockade in the infralimbic prefrontal cortex impaired fear memory extinction (Wei et al. 2012).

Ube3a is a transcriptional coactivator of steroid hormone receptors. Repression of its expression is one of the causes of Angelman syndrome (Sutcliffe et al. 1997). Lack of Ube3a resulted in cognitive and memory impairments, deficits in hippocampal plasticity, seizures, decrease of CaMKII activity, altered adult hippocampal neurogenesis, increased stress and anxiety, and differences in neuronal morphology (Jiang et al. 2010; Mardirossian et al. 2009; Godavarthi et al. 2012; Sato and Stryker 2010; Wallace et al. 2012; Weeber et al. 2003). These phenotypes may be related to defective GR signaling leading to increased stress and anxiety as shown by the fact that mice lacking Ube3a have higher morning corticosterone levels and poor scores in a novel object recognition test and spend more time in the dark (anxiety behavior) in a light/dark test than their wild-type or paternal copy-deficient ube3a mice (Godavarthi et al. 2012). However, as for other coregulators, direct evidence for Ube3a as a mediator of MR and/or GR effects is lacking, and we are at a stage where interactions may be likely, but unproven.

3.4 Discovery of Novel NR Coregulators

Given the effects of coregulators on gene expression and the additional regulation levels they generate, it becomes increasingly more interesting to i) discover new NR coregulators and ii) develop pharmacological agents that can selectively manipulate NR–coregulator interactions.

Finding or predicting new NR coregulators is the first important step, as there is relatively little known about which coregulators interact with which NR. Moreover, even for those coregulators/NRs whose interactions are well documented, little is known regarding their in vivo function in the brain, since the majority of the data comes from in vitro studies. Important data regarding putative interactions between

coregulators and NR may come from the Allen Brain Atlas, where the expression patterns of all NRs and coregulators in the brain have been studied. Furthermore, correlations between the expression of NRs, coregulators, and target genes can take place, providing first hints toward the interactions and involvement of both NRs and coregulators in specific pathways. A second tool to identify putative coregulators with relevance for a particular NR is the MARCoNI peptide array in which receptor–coactivator interactions can be predicted based on NR-box interactions (Desmet et al. 2014; Koppen et al. 2009). With this system, not only the NR–coregulator interactions induced by different ligands can be quantified, but the interactions between coregulators and mutant or recombinant NRs or even the behavior of NRs derived from different in vivo contexts (Houtman et al. 2012). The MARCoNI assay profiles have been previously corroborated in a battery of in vivo tests ranging from stress-related behavior to target gene expression (Zalachoras et al. 2013b). Finally, tools like the MARCoNI assay can also be used in the early stages of drug development to select the better candidates for in vivo use.

For lack of open biochemical approaches based on molecular interactions in small tissues (York et al. 2013), combing data coming from the MARCoNI assay with tools like the Allen Brain Atlas can play an important role in the discovery of novel NR coregulators, predict the behavior and properties of novel NR ligands, and study the properties of NR mutations or modifications.

3.5 Making Use of Coregulator Diversity: Selective Nuclear Receptor Modulators (SNRMs)

Endogenous or exogenous steroids may combine beneficial and disadvantageous effects. Ever since it became clear that there are multiple mechanisms by with the receptors signal, there has been the notion to dissociate such mechanisms with drugs that allow one signaling mechanism, but not others. Such “dissociated compounds” or “selective receptor modulators” that have tissue- or pathway-specific effects may work by several mechanisms, including selective recruitment of coregulators by the receptors (Fig. 3.3). Accordingly, many attempts have been made to develop new drugs with the potential to induce or block selective interactions between NRs and coregulators. Hence, these drugs should induce such an NR-ligand conformation that will make the complex accessible only to a subset of the available coregulators (Martinkovich et al. 2014; Højfeldt et al. 2014).

Most work done on selective androgen receptor coregulators is related to ligands that can target the brain, the bone, or the muscle without affecting prostate tissue with oncogenic potential (Akita et al. 2013). Age-related androgen depletion is a risk factor for sarcopenia, osteoporosis, and accumulation of β-amyloid protein and development of Alzheimer’s disease. Androgen replacement therapies are not always effective due to side effects. The selective androgen receptor modulator NEP28 was shown to increase the expression of an enzyme that breaks down β-amyloid plaques in the brain and was effective in the muscle and bone, without prostate-related adverse effects (Akita et al. 2013). Similar results were also

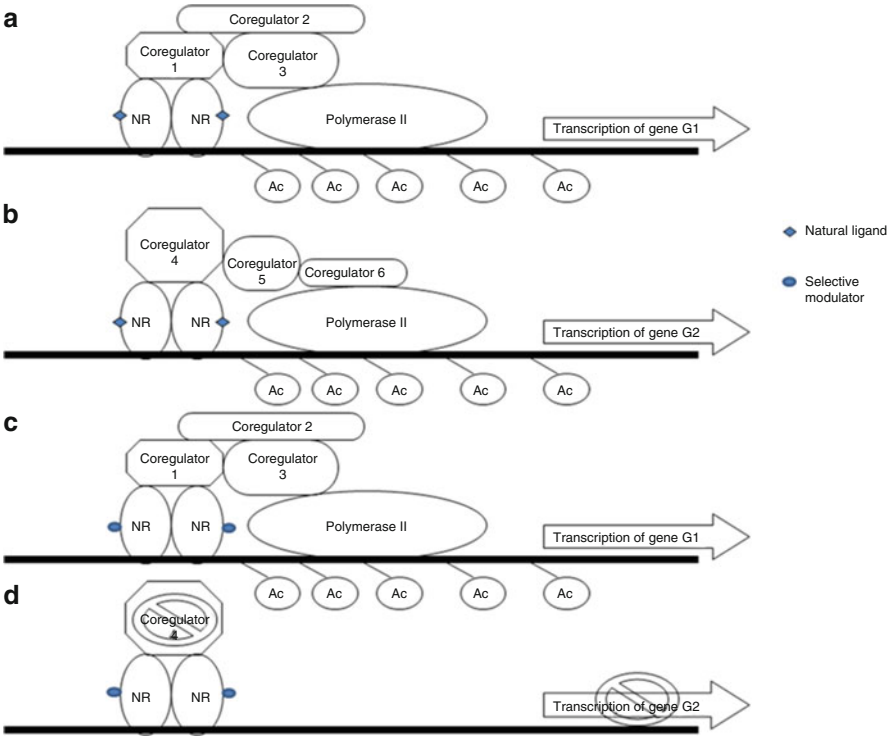

Fig. 3.3 Proposed model of the function of selective modulators. (**a**–**b**). The nuclear receptor is bound to its natural ligand, dimerized, and on chromatin. It can recruit a number of different coregulators that interact directly with it (1,4), which can, in turn, recruit other coregulators (2,3,5, and 6). These NR–coregulator complexes can then stabilize the transcriptional machinery, acetylate histones, and activate the transcription of genes G1 and G2. (**c**–**d**). When NR binds a selective modulator, it only induces/allows interaction with coregulator 1, but not 4. Therefore, only transcription of G1 takes place, while the transcription of G2 is blocked

observed after use of another selective androgen receptor modulator, 3beta,19-NA (Page et al. 2008). Yet another selective AR modulator, A-262536, also showed high selectivity for muscle and bone, in contrast to prostate (Piu et al. 2008). The mechanism of action of these compounds is not fully known; however, at least some of them may induce different AR–coregulator interactions compared to testosterone, while others may capitalize on partial agonist effects or differential penetration of different tissues.

Drugs targeting the ER have a variety of uses including menopausal symptoms, fertility agents or oral contraceptives, and breast cancer treatments (Wardell et al. 2014). Due to the pleiotropic effects of ER in the periphery and the brain, it is important to find agents that have selective action on specific pathways. ER was the prototype target for selective steroid receptor modulators, with tamoxifen, which acts as agonist in the bone and endometrium but as antagonist in the breast (tumors). This selective action was mainly attributed to the selective profile of interactions

between ER and coregulators it can induce, also taking advantage of local expression differences of ER coregulators. Since the development of tamoxifen, additional selective estrogen receptor modulators have been developed with lower side effects and variable ER–coregulator interaction profiles (Feng and O'Malley 2014; Evers et al. 2014; Gottardis et al. 1988). Important for the directionality of the effects of tamoxifen in different tissues are the expression levels of p160 coregulators. Interestingly, increased levels of SRC-1, SRC-3, or other coregulator expression are associated with tamoxifen resistance in breast cancer (Feng and O'Malley 2014; Kumar et al. 2009). Other compounds similar to tamoxifen (nonsteroidal triphenylethylene) are toremifene, droloxifene, and idoxifene all with chemical structure variations in attempts to find the balance between side effects and potency (Martinkovich et al. 2014).

Most attention regarding selective GR modulators has been drawn by GR ligands that have anti-inflammatory efficacy, but no effects on metabolism or osteoporosis (Rauch et al. 2011; van Lierop et al. 2012). However, given the pleiotropic actions of glucocorticoids in the brain, it may be beneficial to distinguish between different effects of glucocorticoids. Blocking detrimental effects of chronically elevated glucocorticoid exposure with full antagonists such as mifepristone can lead to disinhibition of the HPA axis and counteract efficient antagonism. Moreover, blocking all effects of GR on emotional and cognitive processes may not be optimal in order to counteract the negative effects of stress. Similarly, induction in the brain of a pro-inflammatory state by pharmacological blockade of GR in astrocytes and/or microglia may not be desirable. Selective GR or MR modulators may be beneficial in stress-related psychopathology and an interesting and useful tool to distinguish different GR-dependent pathways in experimental settings (Zalachoras et al. 2013c).

First attempts tried to base selective GR modulation on the dissociation of effects that depend on DNA binding by the receptor and classical transrepressive effects directly on pro-inflammatory transcription factors NF-κB and AP1 (De Bosscher et al. 2003). Such an example is the GR ligand "compound A" which induces inhibition of NF-κB-dependent pro-inflammatory transcription, but not DNA binding of GR (De Bosscher et al. 2005; Reber et al. 2012). However, part of the anti-inflammatory effects mediated by GR does depend on binding by GR to classical GREs (Beaulieu and Morand 2011). Coghlan et al. (2003) showed a GR ligand that retained anti-inflammatory effects while preventing the GR effects on glucose metabolism and impact on bones. This study showed that the specific behavior of the compound arose from the GR–coregulator interaction profile it induces. An aryl-pyrazole type of GR ligand was shown to exert selective agonism on hippocampal neurogenesis without affecting skeletal muscle protein synthesis, bone or skin collagen synthesis, or splenic lymphocyte counts (Roohk et al. 2010) and had transcriptional effects on few target genes in cell lines (Wang et al. 2006). This proves the point that GR effects relevant for modulation of brain may be quite selectively targeted with selective modulator types of drugs.

Recently, a novel selective GR modulator has been studied, C108297. It has been shown that it is more specific for GR than mifepristone and can induce a number of GR–coregulator interactions while preventing others. Moreover, it was

shown to have mixed agonist and antagonist properties in stress-related circuits in the brain. For instance, it had agonist effects on the consolidation of fear-related memory, antagonist effects on stress-induced *crh* expression in the CeA, and gene expression in the hippocampus, without inducing HPA axis disinhibition (Zalachoras et al. 2013b). It was also shown that it can counteract the neuroendocrine effects of stress that are induced by glucocorticoid excess (Solomon et al. 2014), as well as prevent the weight uptake as a result of high-fat diet (Asagami et al. 2011). Finally, the same ligand showed strong antagonism that improved the phenotype in animal models of Alzheimer's disease and ALS (Meyer et al. 2014; Baglietto-Vargas et al. 2013).

Selective receptor modulators for MR have not been studied in depth, as plain MR antagonism has been a major clinical goal in cardiovascular disease. However, MR agonism in the brain may be of benefit in relation to particular psychiatric disorders, such as depression (Klok et al. 2011), where its expression has been shown to be decreased in several brain areas (Qi et al. 2012). The development in selective MR modulators is currently taking place, and it will be exciting to see what the potential of such ligands will be (Yang et al. 2011).

3.6 Conclusions

Due to the pleiotropic effects of NRs, modulation of NR-dependent pathways is relevant in a number of conditions. NR coregulators are important for immediate and long-term tissue-, cell-, and target gene-dependent effects of NRs. Therefore, better understanding of NR–coregulator interactions and the development of more selective ligands capable of manipulating those interactions to a desirable direction may be decisive in the treatment of a number of conditions. Although our knowledge has advanced during the past 20 years, there are outstanding questions regarding the gene targets of each coregulator and which protein cocktail is recruited to each particular context. Thus, knowledge of coregulator recruitment to the promoters of certain genes may assist the development of ligands that can affect the expression of genes with high specificity depending on cellular context.

Finally, coregulators can be involved in epigenetic regulation of gene expression either via own activity or via recruitment of appropriate proteins. Thus, studying their epigenetic effects in relation to the changes that appear after a number of environmental stimuli (Elliott et al. 2010; Yehuda et al. 2013; Suderman et al. 2012; Gräff et al. 2014) may reveal new level of regulation and possibilities for intervention.

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Chapter 4
Glucocorticoid-Dependent Epigenetic Regulation of *Fkbp5*

Richard S. Lee

Abstract Emerging evidence suggests that environmental factors, in addition to genetics, may play a crucial role in the development and progression of psychiatric diseases. In particular, chronic exposure to stress or glucocorticoids (GCs) has been shown to elicit symptoms of depression and mania in humans and animals. One of the key modulators of the stress response is a chaperone protein called Fk506-binding protein 5, or FKBP5, that regulates intracellular sensitivity to glucocorticoids. Consequently, genetic variations and epigenetic alterations in the *FKBP5* gene have been linked to depression, bipolar disorder, and PTSD. In animals, chronic exposure to glucocorticoids leads to a persistent loss of *Fkbp5* DNA methylation in the brain. As this epigenetic event mirrors glucocorticoid-induced physiological changes in several organ systems and can be detected in the blood, study of epigenetic regulation of *Fkbp5* may help us understand the underlying mechanism of GC-related psychiatric disorders and the principles that govern the use of peripheral tissues to assess GC-related events occurring in the brain.

Keywords Fk506-binding protein 5 (FKBP5) • Chaperone • DNA methylation • Stress • Glucocorticoids

4.1 Introduction: Genetics, Epigenetics, and the Endocrine System

4.1.1 Genetics of Psychiatric Disorders

For the past two decades, there have been considerable efforts at understanding the etiology of psychiatric disorders at the genomic level. Such efforts started with linkage studies and then moved on to genome-wide association studies (GWAS)

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that sought to identify genetic variations at the base-pair resolution that associated with disease. Now, with more than 100 genome-wide single nucleotide polymorphisms (SNPs) identified in schizophrenia (Ripke et al. 2014) and such similar findings anticipated from other psychiatric disorders, the role of genetics is becoming better documented and understood. The emerging evidence in psychiatric genetics points to psychiatric diseases as complex disorders where numerous SNPs that are relatively common in the population and each SNP accounting for only a small fraction of the disease-relevant "effect" collectively contribute to the classic disorders.

At the same time, despite the high heritability rates of some of the disorders, such as schizophrenia and bipolar disorder (Sullivan et al. 2012), genetics alone cannot completely describe the dynamic nature of these diseases. Age of onset of many of these disorders at early adulthood, cyclical and periodic nature of bipolar disorder, and event-driven disorders such as post-traumatic stress disorder (PTSD) tell us that psychiatric disorders are not strictly borne of the "set-in-stone" genetic blueprint but from a combination of genetics and its complex interaction with dynamic physiological and environmental influences.

4.1.2 Environment

Following the identification of first candidate genes in psychiatric disorders, the field and tools of epigenetics have gained much traction, as they sought to provide the understanding of and the means to describe the functional context in which genetics manifested itself in disease phenotypes. As the term "epigenetics" suggests, its role was to build on top of genetic information to elucidate the conditions that determine gene function. This interplay between genetics and epigenetics has been termed "gene-environment interaction" and seeks to describe the mechanisms by which the cells integrate their gene-determined phenotypes and environmental information.

There are many environmental factors that have been shown to be associated with psychiatric disorders. For instance, nutrition (St Clair et al. 2005; Susser et al. 1996) and prenatal immune activation by infection (Brown et al. 2004; Torrey et al. 2012) or via inflammatory response (Ellman et al. 2010; Nyffeler et al. 2006) have been suggested to be important environmental factors that may play a causal role in schizophrenia. Perhaps the most studied and characterized environmental factor is stress. Stress and its primary agent cortisol, a human glucocorticoid (GC), have been shown to play a significant causal role in mood and anxiety disorders.

4.1.3 Stress, Glucocorticoids, and the Neuroendocrine System

4.1.3.1 Stress, Glucocorticoids, and the HPA Axis

In addition to sex hormones, glucocorticoids are well-studied steroid hormones of the neuroendocrine system. The mammalian neuroendocrine system consists of peptides and hormones and their associated tissues that mediate our internal

development as well as our interaction with the environment. In particular, the HPA axis system consisting of the hypothalamus, pituitary, and adrenal glands initiates the production and termination of the stress glucocorticoid cortisol that mounts our primary stress response. So-called the fight or flight response, the stress response of the HPA axis is triggered when stressors are perceived by the brain, and this leads to the release of cortisol and adrenaline from the adrenal glands. While the tightly regulated, adaptive negative feedback system returns the increased levels of cortisol down to baseline, a failure to do so due to prolonged exposure to the catabolic effects of cortisol has maladaptive consequences. The maladaptive consequences of excessive exposure to stress can be seen in the modern society of the twenty-first century, where complex social behavior has led to many stress-related diseases at epidemic proportions.

The Sapolsky studies on primates (Sapolsky 1982, 1989) and the Whitehall II study (also known as Stress and Health Study) (Marmot et al. 1991) exemplify the detrimental role of psychosocial stress and its impact on the organism, where occupation of positions of "lower rank" was associated with a host of ailments including cardiovascular disease (Steptoe and Willemsen 2004), metabolic syndrome (Brunner et al. 1997), and psychiatric disorders (Stansfeld et al. 1997, 1999).

In particular, the brain is especially susceptible to prolonged exposure to stress and its primary glucocorticoid cortisol. Glucocorticoid (GC) exposure in its various duration and intensity has been associated with mania, depression, and suicide in a landmark epidemiological study that has reported a significant increase in these psychiatric outcomes in exposed individuals (Fardet et al. 2012). In addition, exposure to intense stressors, in the case of trauma, often leads to symptoms of PTSD (Yehuda et al. 2010). Whether it be child abuse, sexual assault, or military combat, PTSD is an HPA axis-related anxiety disorder marked by reduced fear extinction and dysregulated GC regulation (McFarlane et al. 2011; Norrholm et al. 2011).

One human condition that offers a great insight into the causal role of glucocorticoids on mental health is Cushing's disease, a "rare" disease affecting 10–15 million people annually, where in one type of Cushing's, pituitary tumors cause hypersecretion of ACTH that subsequently leads to excessive cortisol production. In Cushing's disease, a substantial number of patients (60–90 %) develop depressive symptoms that become mitigated following surgical removal of the tumor and resolution of hypercortisolemia (Cohen 1980; Dorn et al. 1997; Flitsch et al. 2000; Starkman et al. 1986). Cushing's disease serves as a useful human model to understand the causative role of glucocorticoids in psychiatric diseases.

4.1.3.2 HPA Axis Genes

How does prolonged or intense exposure to glucocorticoids contribute to psychiatric symptoms? Glucocorticoids, and more specifically cortisol, bind to a member of a family of nuclear hormone receptors called the glucocorticoid receptor (GR or NR3C1) that translocates to the nucleus and acts as a transcription factor. Of the many genes that are either activated or silenced by GR, they can simplistically be categorized into two groups based on their relationship to glucocorticoid levels

and cellular function. One type is made up of those genes that can directly impact the levels of cortisol by affecting its production, degradation, and intracellular signaling. The glucocorticoid receptor (*GR*), FK506-binding proteins 5 and 4 (*FKBP5* and *FKBP4*, respectively), corticotropin-releasing hormone (*CRH*), and pro-opiomelanocortin (*POMC*) are such genes whose proteins are capable of affecting steady-state levels of systemic GCs and intracellular GR signaling. Peptide hormones encoded by genes such as *CRH* and *POMC* are involved in the negative feedback mechanism of the HPA axis and can directly influence overall cortisol homeostasis, while proteins such as GR, FKBP5, and FKBP4 have been shown to modulate intracellular glucocorticoid signaling. These intracellular proteins can also affect systemic plasma cortisol levels by preventing cortisol from participating in GR signaling and causing its extracellular accumulation. Examples of this can be observed in primates, where gene mutation studies have shown that the New World monkeys exhibit hypercortisolemia and glucocorticoid resistance due to an enhanced affinity of FKBP5 for GR (Denny et al. 2000; Reynolds et al. 1999), and in vitro, where FKBP5/FKBP4 ratios determine the strength of GR signaling into the nucleus (Wochnik et al. 2005). Given the constellation of symptoms caused by prolonged exposure to glucocorticoids and stress, it can be surmised that many types of tissues are affected in such way, and these signals at different tissues collectively contribute to hypercortisolemia and glucocorticoid resistance.

A potential role for these types of genes in psychiatric disorders comes from numerous candidate gene association studies that have linked SNPs in these genes with psychiatric disorders or clinical studies that show dysregulation of cortisol dynamics in patients with psychiatric disorders. For instance, studies that have examined normal physiological cortisol levels, such as the cortisol awakening response, or the ones designed to challenge the HPA axis negative feedback system, such as the dexamethasone suppression test, have found abnormal HPA axis function in PTSD (de Kloet et al. 2007; McFarlane et al. 2011; Wessa et al. 2006) and depression (Jarcho et al. 2013; Vreeburg et al. 2009). These findings suggest that perturbations in the HPA axis system and cortisol homeostasis may precede or sustain disruptions in specific neuronal processes associated with psychiatric disorders.

The second group of HPA axis genes consists of target genes that are specific for tissue function. For instance, GC target genes such as *BDNF* (Barbany et al. 1992), *TH* (Rani et al. 2009), and *PER1* (Reddy et al. 2012) may be relevant to brain function and neurotransmission, while genes encoding cytokines, such as interleukins 7 and 13, may be more relevant for the immune system (Blanchard et al. 2007; Takeda et al. 1998). Stress-related psychiatric diseases then can be seen as a result of prolonged activation or silencing of neuronal genes caused by a combination of prolonged exposure to glucocorticoids and its ensuing maladaptive HPA axis function through alterations of genes that directly alter cortisol metabolism, which often continue to persist even in the absence of additional GC signaling.

4.1.3.3 Glucocorticoids, HPA Axis Genes, and Epigenetics

If one of the primary roles of GC signaling is to cause changes in gene activity, then what is the mechanism by which stress and glucocorticoids persistently alter HPA axis gene function and ultimately affect a person's internal cortisol dynamics and behavior? The underlying mechanism of such a role for glucocorticoids can be attributed to the field of epigenetics. As epigenetics is a measurement of a gene's transcriptional potential or inertia by assessment of its structural context, it provides the means to how genes become activated or silenced over time. Through mechanisms that are recently coming to light, glucocorticoids have the ability to cause long-lasting changes in the structures and modifications of DNA and histones that lead to alterations in the genes' ability to undergo transcription.

In fact, central to how epigenetics plays a role in psychiatric diseases through the neuroendocrine system may be the ability of glucocorticoids to directly alter epigenetic marks, either on DNA or the histone proteins that the DNA is wrapped around. In the past decade, numerous studies have documented significant changes in the epigenetic marks on HPA axis genes following exposure to stress. Work on *Crh* (Elliott et al. 2010), *Gr* (Weaver et al. 2004), and *Bdnf* (Roth et al. 2009; Tsankova et al. 2006), among others, shows that stressors imposed at different developmental periods lead to alterations in DNA methylation or histone acetylation at these genes, consistent with their transcription level changes. However, as stress response consists of additional hormones such as adrenaline, only a few studies have directly demonstrated the role of glucocorticoids in epigenetics.

Perhaps one of the earliest evidences of a direct, causal role of glucocorticoids and epigenetics was demonstrated in the gene that encodes the tyrosine aminotransferase (TAT), a well-known target gene of glucocorticoids (Nitsch et al. 1993). Using rat fetal liver tissues and a rat liver cell line, a noteworthy study demonstrated that exposure to glucocorticoids leads to a persistent change in DNA methylation, thus providing a "memory of the first stimulation" (Thomassin et al. 2001). Ability of glucocorticoids to alter DNA methylation in a mouse model was demonstrated in the *Fkbp5* gene (Lee et al. 2010), where administration of the mouse glucocorticoid corticosterone (CORT) for 4 weeks caused persistent decrease in DNA methylation near GC response elements (GREs), which lasted for additional 4 weeks following withdrawal of glucocorticoids. This finding was recapitulated in the mouse neuronal cell line HT-22, where 1-week treatment with CORT caused dose-dependent, persistent changes in DNA methylation in the same CpG dinucleotides as in the mouse. A more recent work demonstrated that the primary agents responsible for stress-induced epigenetic changes are indeed glucocorticoids (Niwa et al. 2013). In this work, the authors demonstrated that administration of the glucocorticoid receptor antagonist RU38486 (or mifepristone) during the stress regimen was able to prevent the neurochemical, behavioral, and epigenetic deficits observed in saline-treated animals.

4.2 *Fkbp5:* Epigenetic Mechanisms and Disease

4.2.1 Fkbp5 and Glucocorticoid Signaling

As mentioned, one of the HPA axis genes that can potentially alter cortisol dynamics through DNA methylation is *Fkbp5*. It encodes a chaperone protein, which together with the heat shock proteins HSP70 and HSP90, sequesters the unbound glucocorticoid receptor (GR) (Grad et al. 2007). In the presence of glucocorticoids, the GR disassociates from the FKBP5/HSP70/HSP90 complex and translocates into the nucleus via interactions with FKBP4 and members of the dynein family of motor proteins (Wochnik et al. 2005). One of the immediate early target genes of nuclear GR signaling is *Fkbp5* itself, which upon transcription and translation resides in the cytoplasm to reinforce the heat shock chaperone complex and to diminish GR signaling (Fig. 4.1). This intracellular negative feedback has significant implications for intracellular GR signaling as well as extracellular levels of cortisol. As mentioned previously, work in primates (Denny et al. 2000; Reynolds et al. 1999) showed that hypercortisolemia and glucocorticoid resistance can be attributed to the FKBP5 protein in squirrel monkeys that has enhanced affinity for GR. In humans, genetic variations in *FKBP5* have been implicated in depression, antidepressant response (Binder et al. 2004), bipolar disorder (Willour et al. 2009), PTSD (Binder et al. 2008; Roy et al. 2010), and even in Alzheimer's disease (Blair et al. 2013; Sabbagh et al. 2014), where changes in FKBP5 levels are associated with known risk factors of Alzheimer's, such as stress exposure, tau formation, and aging.

For the second half of this chapter, we will focus on what our work on the mouse *Fkbp5* gene has taught us in the context of glucocorticoid exposure, epigenetics, and psychiatry-relevant behaviors that will support clinical findings in the human *FKBP5* gene discussed elsewhere in this book.

4.2.2 Loss of DNA Methylation

4.2.2.1 Epigenetic Changes at Glucocorticoid Response Elements

One of the main findings from studying *Fkbp5* is that glucocorticoids can directly alter DNA methylation. This phenomenon was first demonstrated in the rat *Tat* gene (Thomassin et al. 2001) and recapitulated on the mouse *Fkbp5* gene in an animal model of excessive CORT exposure and in cell culture (Lee et al. 2010). Not surprisingly, the locations of these sites of epigenetic changes correspond to binding sites for the glucocorticoid receptor dimer (or GRE, glucocorticoid response element), just as many of the genes implicated in stress and glucocorticoid-related behavioral deficits that are known target genes of glucocorticoids harbor GREs. For instance, *Bdnf*, *Th*, *Crh*, *Sgk1*, *Fkbp5,* and *Gr* are all genes important for psychiatric disorders, have been identified as target genes of glucocorticoids, and all contain GREs (Hagerty et al. 2001; Hubler et al. 2004; Itani et al. 2002; King et al. 2002;

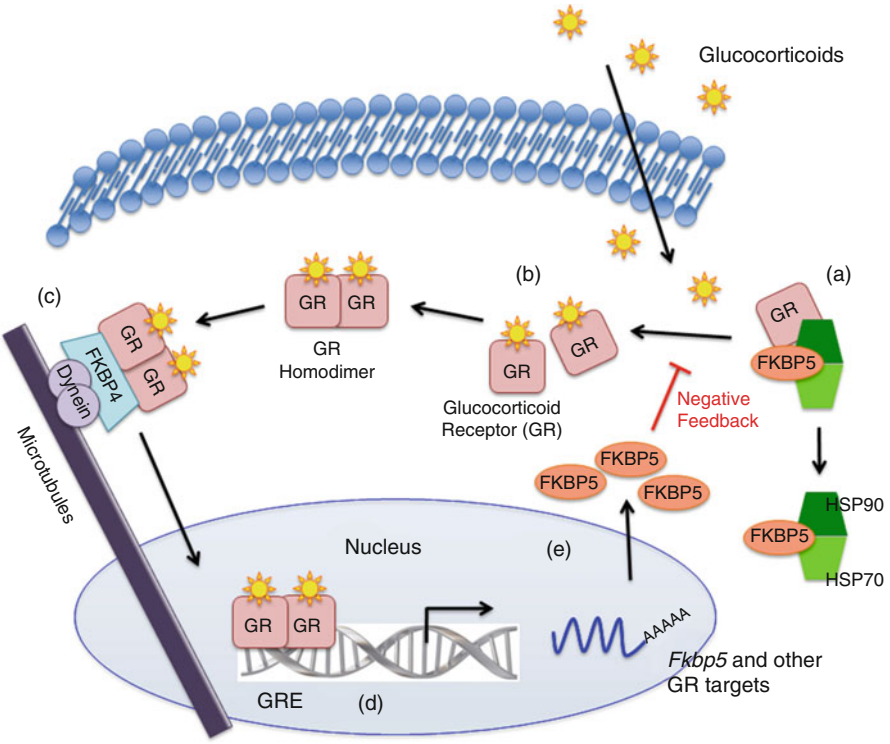

Fig. 4.1 Intracellular glucocorticoid receptor signaling and FKBP5. (**a**) The glucocorticoid receptor is a nuclear hormone receptor that resides in the cytoplasm bound to the chaperone complex consisting of FKBP5, HSP90, and HSP70. (**b**) Upon glucocorticoid (cortisol) binding, the glucocorticoid receptor (GR) dissociates from the chaperone complex and homodimerizes. (**c**) The homodimer interacts with the FKBP4-dynein complex and undergoes microtubule-assisted nuclear translocation. (**d**) Once in the nucleus, the GR homodimer binds to DNA at palindromic repeats known as glucocorticoid response elements (GREs). (**e**) One of the many genes that are regulated by GR is the *FKBP5* gene that after transcription and translation hinders further dissociation of GR from the chaperone complex. Dysregulation of this intracellular negative feedback loop is thought to be largely responsible for glucocorticoid resistance and hypercortisolemia

Ramamoorthy et al. 2013; Wosiski-Kuhn et al. 2014). Epigenetic changes observed at these genes provide the focal point that transduces the environmental stimulus via stress and glucocorticoids into stable modifications on DNA and histones that affect gene expression, neuronal processes, and, ultimately, behavior.

4.2.2.2 Potential Mechanism of GR-Mediated Epigenetic Changes

How the complex consisting of the GR dimer and unknown cofactors achieves loss of DNA methylation at *Fkbp5* is mostly undetermined. Although this process will most likely involve one or more of the methyltransferases such as DNMT1 (Crudo

et al. 2012; Yang et al. 2012), any of the recently characterized enzymes capable of demethylation such as TET1 (Guo et al. 2011), GADD45B (Ma et al. 2009), the mismatch base excision machinery (Santos et al. 2013), replication of neural progenitor cells (Ohno et al. 2013), or a combination of these mechanisms, very little work has been done in the context of glucocorticoid-mediated loss or gain of methylation.

When considering the potential epigenetic mechanisms employed by glucocorticoids, there exists an apparent paradox in that glucocorticoids can both activate and repress transcription. This has been shown by numerous candidate gene as well as genome-wide transcriptome studies. For instance, while there exist a number of genes upregulated by glucocorticoids such as *Tat*, *Fkbp5*, and Sgk1, among others (Polman et al. 2012), there are also a vast number of genes whose expression is repressed by GR (Surjit et al. 2011). If GR binding to genes such as *Fkbp5* causes loss of DNA methylation, then how can the same GR complex recruit factors that promote increase in DNA methylation, as observed at genes silenced by GR signaling, such as *Gr* itself (Weaver et al. 2004) and *Bdnf* (Roth et al. 2009)?

Likewise, a potential paradox also exists in studies that have attempted to identify changes in levels of intracellular epigenetic machinery in the context of glucocorticoid exposure and transcription. Some have reported changes in the levels of DNMTs (DNA methyltransferases) or histone-modifying enzymes such as HDACs (histone deacetylases) that may potentially offer a consistent mechanistic explanation of changes in gene function observed in a particular study. For instance, we have observed a significant dose-dependent decrease in the levels of the *Dnmt1* gene in the AtT-20 pituitary cell line and in the mouse hippocampus treated with glucocorticoids (Yang et al. 2012). While this may be consistent with the loss of DNA methylation and *increase* in expression of *Fkbp5*, it is inconsistent with the observed GC-induced *decrease* in expression levels of genes such as *Crh*, *Gr*, or *Bdnf*, where DNMT1 protein should be higher if DNA methylation plays a role. Conversely, other groups have also noted additional epigenetic machinery, such as histone deacetylating enzyme HDAC1 and transcriptional repressor MeCP2, associated with GR-mediated *silencing* of *Crh* (Sharma et al. 2013). How can the same GR complex associate with epigenetic-modifying enzymes that can activate or repress transcription, depending on the target gene? An answer may be found in a novel mechanism, most likely common among nuclear hormone receptors, of gene regulation by GR, where DNA sequence may alter the structure of the bound GR dimer (Meijsing et al. 2009). Sequence-directed structural and activity changes in GR may distinguish the types of transcription factors and repressors and different epigenetic-modifying enzymes that it can associate with at the GREs. In other words, it is likely to be the case that the appropriate histone-modifying or DNA methylation-altering enzymes are recruited to specific genomic loci based on altered structure of the GR complex bound to its specific GRE. In addition, this GRE-specific recruitment of factors by GR may occur independently of global changes in the levels of the enzymes that modify DNA or histones.

4.2.2.3 Effect of DNA Methylation on Transcription

Fortunately, in contrast to the epigenetic mechanism of gene regulation by glucocorticoids, mechanism of GR-mediated gene regulation is better understood. GR-mediated gene regulation occurs with binding of the GR dimer to its GRE, which is often located in intronic regions (Polman et al. 2013), then proceeds with recruitment of enhancer/transcription factors and looping of the intronic GRE DNA to the promoter, and concludes with initiation or suppression of transcription (Fig. 4.2a, c). While the looping of the GREs to their respective promoters of *FKBP5* and *GR* has been elegantly demonstrated (Klengel et al. 2013; Ramamoorthy et al. 2013), it is yet unclear as to the role of loss or gain of non-promoter DNA methylation on gene regulation. While evidence so far suggests a more robust activation of transcription, as in the case of *Tat* (Thomassin et al. 2001) and *FKBP5* (Klengel et al. 2013), how loss of DNA methylation of regions that are often tens of kilobases away directly influence transcription is still a mystery. We posit that loss of DNA methylation in the case of *Fkbp5* and presumably concomitant increase in euchromatic marks and nucleosomal remodeling near the GRE (Paakinaho et al. 2010) affect the speed of loading of methylation-sensitive enhancer factors which in turn affects the strength of the GRE-promoter interaction. As chronic exposure to glucocorticoids "wears down" DNA methylation at the *Fkbp5* locus, it increases the likelihood of the region becoming occupied by enhancer factors and decreases its occupation by methylation-dependent repressors (Fig. 4.2b). This in turn enables a quicker response time and more robust transcriptional activation, as more cells become engaged in transcription in a shorter period than those with higher DNA methylation and less occupation of enhancer factors (Fig. 4.2d). One of the evidences that supports the binding of methylation-sensitive factors near these areas of methylation change is that a GRE within *Fkbp5* that undergoes loss of methylation in the blood coincided with a binding site for MeCP2 (Nuber et al. 2005), a potent methylation-sensitive repressor. The role of MeCP2 in stress-mediated silencing of other HPA axis genes has also been demonstrated at the *Pomc* locus (Wu et al. 2014).

4.2.3 Persistence of Epigenetic Changes

Of great interest and relevance to the field of psychiatry is the observation that GC-caused DNA methylation patterns can persist throughout development. In the case of *Tat*, glucocorticoid-induced loss of methylation persists and retains the "memory" of the exposure throughout embryonic liver development (Thomassin et al. 2001). In the case of *Fkbp5*, DNA methylation in the *Fkbp5* gene caused by rodent glucocorticoid corticosterone (CORT) exposure during adolescence also persisted into adulthood long after CORT withdrawal (Lee et al. 2010). This phenomenon was recapitulated in a simple cell line model, where CORT-induced loss of methylation also persisted well into several weeks following its withdrawal.

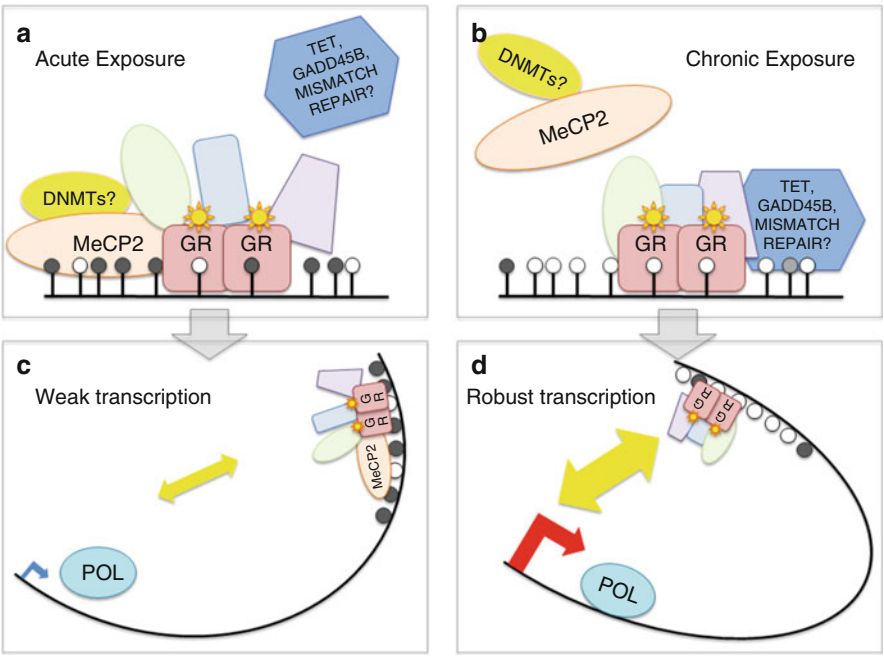

Fig. 4.2 Effects of DNA methylation on expression of *FKBP5*. (**a**) During acute exposure to stress or glucocorticoids, the GR homodimer binds to the GREs, along with yet unidentified methylation-sensitive enhancer factors or methylation-dependent repressors such as MeCP2. The native DNA methylation patterns at or near the GREs influence the binding affinity of these enhancers and suppressors. Following transcription initiation, the GR complex dissociates from the GREs. (**b**) With chronic glucocorticoid exposure, prolonged presence of the GR complex at the GRE may decrease DNA methylation by recruiting DNA demethylating enzymes such as TET, GADD45B, or the mismatch base excision machinery. Alternatively, occupation of the GREs by GR may prevent DNA methyltransferases (DNMTs) from maintaining DNA methylation. (**c**) Transcriptional activation of the GR-enhancer complex following acute glucocorticoid exposure involves interaction between the intronic GREs and the promoter, ultimately resulting in transcription by the polymerase complex (POL). Strength of the interaction, and hence *FKBP5* expression, is determined by the binding of enhancers at the GREs. (**d**) With prolonged glucocorticoid exposure, loss of DNA methylation promotes greater enhancer factor loading at the GREs, which allows for a stronger interaction between the promoter and the GREs. Faster *FKBP5* transcription in GR-sensitive cells collectively leads to robust FKBP5 production, reduction in additional GR signaling, and glucocorticoid resistance

This persistent effect may have significant implications for the mammalian brain. The ability of the cells to sustain and retain glucocorticoid-induced damage for long periods of time may underlie the persistence and chronicity of stress- and glucocorticoid-related psychiatric disorders. It is tempting to speculate that persistence of stress-related disorders may be tied to this very nature of glucocorticoids. For example, animals and humans exposed to such epigenetic insults at the *FKBP5* locus may achieve a quicker and more robust expression of *FKBP5* and therefore experience heightened and prolonged hypercortisolemia and glucocorticoid resistance.

Similarly, those that harbor increased DNA methylation of *GR* or *Bdnf* are likely to experience a similar reduction in GC signaling and dysregulated neuronal function, respectively. Although the picture is not yet clear, these implications have gained empirical support from both clinical (Klengel et al. 2013) and animal model (Weaver et al. 2004) studies.

4.2.4 Measurement of Glucocorticoid Exposure

One of the testable implications of the observation that glucocorticoids can persistently alter DNA methylation of *Fkbp5* was the possibility that degree of methylation can hold information on the amount (concentration x duration) of exposure to glucocorticoids. Although it is highly unlikely that epigenetic alterations at any single HPA axis gene can serve as a marker for complex disorders such as depression or bipolar disorder, it seems feasible that DNA methylation can be used to assess glucocorticoid exposure. While cortisol can be readily measured from saliva, hair, urine, or blood and serve as an indicator for overall stress exposure, it is a highly pulsatile hormone whose large intra-subject fluctuations in a single day and over days and months (Hansen et al. 2001) due to physiological, psychological, or pharmacological processes render single samplings inaccurate and insufficient. Given the observation that glucocorticoids can directly alter DNA methylation, a relatively stable modification, epigenetic changes at the GRE of *Fkbp5* in blood were compared against a 1-month glucocorticoid exposure measurement determined by taking daily and weekly serial measurements of plasma CORT. There was a significant correlation between DNA methylation of *Fkbp5* in the blood vs. overall CORT exposure. Further, there were significant relationships with other parameters associated with chronic glucocorticoid exposure, such as atrophy of the thymus and adrenal glands, increase in visceral fat, and anxiety-like behavior (Lee et al. 2011). These preliminary results suggested the utility of *Fkbp5* DNA methylation measurements as an alternative to serial measurements of CORT, which can be cumbersome, and as a potential proxy to glucocorticoid-caused physiological changes occurring in less accessible tissues in the body.

4.2.5 Heterogeneity of Tissues

One of the challenges encountered during the study of *Fkbp5* methylation was in overcoming the relatively small effect sizes observed in DNA derived from the whole hippocampal tissues (Lee et al. 2010). The presence of more substantial DNA methylation changes in a cell line treated for only 1 week (compared to 4 weeks in mice) raised the possibility that one of the factors that may affect observable effect size was tissue heterogeneity. In subsequent experiments, efforts were made to enrich for granule neurons of the hippocampal dentate gyrus by performing small

hole punches in lieu of gross dissections. Implementing this crude procedure was able to double the observable DNA methylation changes and implied that there are compartments in the brain, presumably neuronal, which undergo greater GC-related epigenetic changes than nonneuronal compartments. Now there are numerous approaches, some experimental methods such as fluorescence-activated cell sorting (FACS) (Jiang et al. 2008) and others using statistical methods (Houseman et al. 2012), to address the dilution effect posed by tissue heterogeneity in many biological samples. A study that exemplifies the need to isolate homogeneous population of cells for epigenetic analyses employed retrograde beads injected at the frontal cortex and the nucleus accumbens to label dopaminergic neurons of the ventral tegmental area (VTA), followed by the use of the fluorescence from the beads and FACS to isolate different projection-specific neuronal populations. Using this method, substantial GC-caused epigenetic changes in the tyrosine hydroxylase (*Th*) gene, but only in the VTA neurons that projected to the cortex, were observed (Niwa et al. 2013).

4.2.6 Across-Tissue Correlation

There are now numerous studies that have identified putative biomarkers of psychiatric disorders, where some peripheral biological measures such as blood DNA methylation, SNPs, or gene expression are correlated with disease status or severity of symptomatology. While it may be easy to dismiss the notion that some changes in a peripheral source can mirror disease-relevant changes in the brain, many clinical studies have been predicated and published on this assumption and thus necessitate experimental validation. However, efforts to prove that peripheral biomarkers can serve as a surrogate to psychiatric disorders are exceptionally challenging without first knowing the nature of the pathological process linking the biomarker and the disease. On the other hand, it may be more feasible to identify markers that can predict the activity of specific physiological processes that underlie a psychiatric disorder rather than the specific psychiatric disorder itself. For instance, dexamethasone-treated blood cells have been used to predict glucocorticoid resistance in depression patients (Menke et al. 2012).

For *Fkbp5* in mice, the observation that glucocorticoids induced dose-dependent changes in DNA methylation in blood raised the possibility of making similar observations in the brain and deriving a correlation between these two measurements, with the goal of using blood methylation to provide information on GC-induced changes in the brain. In a proof-of-concept experiment, DNA methylation measured in the brain and blood of mice that were treated with different doses of CORT was first compared to mean plasma CORT levels from four serial, weekly samplings. The robust correlation between blood methylation and plasma CORT levels and a modest one between brain methylation and plasma CORT were based on the existence of a common mechanism, i.e., functioning GC signaling as

indicated by *Fkbp5* induction and dose-dependent changes in DNA methylation that existed in both types of tissues. DNA methylation values from blood showed modest correlation to hippocampal DNA methylation and expression of *Fkbp5*, suggesting that a small sampling of blood DNA methylation can serve as a peripheral, accessible proxy to methylation and expression in the brain and obviated the need for cumbersome serial measurements of plasma glucocorticoid levels (Ewald et al. 2014). The presence of such a correlation between DNA methylation in the blood and gene expression in the brain suggested the possibility of being able to assess the degree of *Fkbp5*-mediated glucocorticoid resistance in the hippocampus by sampling blood DNA methylation. Further, this experiment raised the possibility of additional targets and genes in the blood that may be used to predict functions of other specific processes in the brain.

4.3 Perspectives and Future Directions

4.3.1 Mechanism of Epigenetic Changes

Studying the role of an important modulator of HPA axis function such as *Fkbp5* has provided an insight into the role of epigenetics in glucocorticoid-related psychiatric disorders. By studying the epigenetic regulation of *Fkbp5* in the brain and blood, we have learned the following: (1) DNA methylation can be altered directly by GCs, lending itself as an excellent indicator of environmental stressors; (2) methylation marks can persist across developmental periods; (3) inertial change of DNA methylation serves as a robust indicator of exposure history in mice; (4) tissue heterogeneity poses a significant obstacle for effect size determination; and (5) across-tissue correlates may exist for glucocorticoid target genes between different tissues.

However, there are great gaps in knowledge and questions that must be addressed. For instance, the mechanism of loss or gain of DNA methylation is largely unknown, as well as the key factors that mediate its process. Identification of the mechanism and factors may provide the key to understanding the chronicity and persistence of stress- and trauma-related psychiatric disorders.

4.3.2 Additional Targets of Glucocorticoids

Second, it is also important to identify additional targets of glucocorticoids. As mentioned here with *Fkbp5* and elsewhere with other genes of the neuroendocrine system, there are only a handful of genes that have been studied in great detail. A mechanistic understanding of additional GC targets may provide more insight to the underlying pathologies and pathways that affect different psychiatric disorders.

4.3.3 Medications to Alter Glucocorticoid Signaling

Third, accumulating evidence that implicates HPA axis dysregulation in the etiopathology of psychiatric disorders raises the potential use of agonists and antagonists of GR and its associated chaperone factors as medications to combat psychiatric illnesses. Currently, GR antagonists such as mifepristone are available as potential therapeutic medications for psychotic depression. In an animal model, administration of mifepristone was able to prevent many of the stress-induced neurochemical, epigenetic, and behavioral changes in the brain (Niwa et al. 2013). Nevertheless, these medications are not without considerable side effects, due to their potency, cross-reactivity with other nuclear receptors, and immunosuppressive effects. Therefore, with a better understanding of the mechanism and the key interacting players involved, it may be possible to develop medications that specifically target disease-relevant processes and can uncouple therapeutic benefits from the undesirable side effects.

4.3.4 Prediction of Glucocorticoid-Induced Pathologies Using Peripheral Markers

Lastly, proof-of-concept work performed on the *Fkbp5* gene in the blood and brain raises the exciting possibility of being able to assess brain-specific events and measurements through the use of blood epigenetic markers. There are already tools and methods to measure various parameters in patient brains, such as imaging studies using isotope-tagged tracers and for measuring brain volumes. However, it might be possible to use peripheral epigenetic patterns to estimate the GC-induced changes in assessing the function of specific genes and processes in the brain. For example, identification of epigenetic changes in the blood tyrosine hydroxylase gene and the existence of a strong correlation between those measurements in the blood and brain may allow us to assess GC-related changes in dopamine function in the brain.

4.4 Summary

In this chapter, we examined the role of glucocorticoids in psychiatric disorders through epigenetic control of HPA axis function. Epigenetic studies performed on genes such as *Fkbp5*, among other key HPA axis genes, have provided valuable information on how prolonged exposure to glucocorticoids can lead to specific pathological processes in the brain. As the field of psychiatric epigenetics continues to grow, additional tools and approaches are needed to identify key epigenetic factors, to characterize peripheral predictive biomarkers, and to develop more target-specific medications.

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Chapter 5
Epigenetic Programming of the HPA Axis by Early Life Adversity

Christoph A. Zimmermann, Florian Raabe, and Anke Hoffmann

Abstract Early life adversity (ELA) is an important risk factor for the manifestation of various psychiatric disorders later in life. Prenatal and postnatal brain development comprises a time of heightened neuronal plasticity, and ELA during these periods can lead to long-lasting changes in the formation of neuronal activity-dependent circuitries and structures in conjunction with epigenetic marking of genes important to cognition, mood, and behavior. Among various epigenetic marks, DNA methylation is the best characterized besides histone modifications and chromatin remodeling factors. ELA during critical windows of development can cause sustained deregulation of the hypothalamic-pituitary-adrenal (HPA) axis, a major mediator of the stress response, whose deregulation during the course of major depression is thought to recall ELA exposure.

ELA in mice evokes epigenetic programming of the hypothalamic neuropeptide arginine vasopressin (*Avp*), a critical driver of the HPA axis, with the epigenetic reader methyl-CpG-binding protein 2 (Mecp2) playing an important role in the establishment and maintenance of ELA-dependent epigenetic marks. Similarly, Mecp2 mediates epigenetic programming of pituitary pro-opiomelanocortin (*Pomc*), a downstream effector of the neuronal stress response. Overall, these studies suggest that Mecp2 integrates ELA at different levels of the HPA axis and point to the need for timely therapeutic interventions to prevent the progression of potentially harmful epigenetic marks.

Keywords Early life adversity • Early life stress • Major depression • Epigenetic programming • HPA axis • Mecp2

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5.1 Introduction

The prevalence of psychiatric diseases continued to rise over the past decades with mental and substance use disorders increasing by more than one-third between 1990 and 2010 (Degenhardt et al. 2013). Depressive disorders place the highest burden on society when measured in years lived with disability and affect most strongly adolescents and young adults (Degenhardt et al. 2013). While psychiatric diseases are not among the deadliest (such as cancer), their impact on society remains tremendous, and worryingly though, research funding makes up only a fraction of the one granted to other common diseases such as cancer (Ledford 2014).

Traumatic events in early life are well-recognized risk factors for later psychiatric diseases. In humans, a childhood history of abuse, trauma, or neglect has been clearly shown to increase the susceptibility for the development of affective disorders later in life (Heim and Nemeroff 2001; Heim and Nemeroff 2002; Lupien et al. 2009; Green et al. 2010). Despite a slight decrease for the past 5 years in the overall rates of child victimization and in death from maltreatment (U.S. Department of Health and Human Services et al. 2013), current trends from various forms of child maltreatment remain alarming. In 2012, an estimated 3.5 million referrals involved the alleged maltreatment of approximately 6.4 million children in the USA. Approximately one-fifth of the children investigated were found to be victims of abuse or neglect. This rate corresponds to 9.1 per 1000 children in the population whereby children in the age group of birth to 1 year suffered from the highest rate of victimization at 23.1 per 1000 children. In accord with prior years, neglect was overwhelmingly the most common form of child maltreatment (79.5 %) followed by physical (18 %) or sexual (9 %) abuse and psychological maltreatment (8.7 %).

Even so strong evidence has accumulated for ELA's impact on future mental health, the underlying molecular mechanisms connecting adverse early life experiences to intermediate risk phenotypes (Hornung and Heim 2014), and ultimately later disease, remain presently poorly understood (Hoffmann and Spengler 2012).

In this regard, a series of recent experiments in mice has provided new insight into the critical involvement of various levels of the HPA axis in the process of coupling early life experiences via epigenetic mechanism to stress susceptibility later in life. As an import regulator of HPA axis activity, the stress gene *Avp*, encoding the neurohypophyseal peptide arginine vasopressin, has been shown to be epigenetically programmed by ELA. A two-step process has been proposed where Mecp2 phosphorylation leads to a decrease in enhancer methylation and associates with a sustained increase in *Avp* expression (Murgatroyd et al. 2009). At the same time, Mecp2 has been shown to contribute to the epigenetic marking and overexpression of pituitary *Pomc*, a downstream target of Avp signaling (Wu et al. 2014).

In this chapter, we will discuss current advances in the understanding of how adverse early life experiences can influence gene expression through an interaction with epigenetic mechanisms and summarize latest findings of Mecp2's integrated role in epigenetic programming of the HPA axis.

5.2 The Impact of Early Life Experiences on the (Epi-) genome

It is well known that some individuals are more susceptible to various stressful experiences than others, and these differences in vulnerability are thought to be explained by differences in their genetic background as well as in their lifetime history of adverse but also favorable experiences and, ultimately, how both genes and environment conspire together.

5.2.1 Windows of Vulnerability

All living beings are exposed to different kinds of challenges and threats that differ in quantity and quality with outcomes potentially accumulating across lifetime (McEwen 2007; Lupien et al. 2009). An individual's organism is thought to respond to such stressors with immediate and long-term adjustments in different physiological systems to reinstate homeostasis. Importantly, there are critical stages during lifetime where an individual is more susceptible to such exposures (Fig. 5.1). This applies in particular to the brain that undergoes periods of neuronal (re)wiring and (re)organization not only prenatally but also postnatally throughout childhood to puberty and is furthermore subjected to age-related changes in adult and aged individuals. The strongest influence of stress can be observed in brain regions that are developing at the time of stress exposure (Lupien et al. 2009). Adverse stressful life experiences, but also positive master experiences, can change brain circuitries via activity-dependent plasticity and induce permanent structural and regulatory changes (Katz and Shatz 1996). The development of brain regions like hippocampus, frontal cortex, and amygdala, all of which regulate HPA axis activity, is especially affected by stress in the perinatal period with alterations in stress responses frequently persisting into adulthood. Since the frontal cortex additionally undergoes major refinements during adolescence, stress during this time interval may have negative effects as well, while other brain regions susceptible to the effects of aging may preferentially suffer from stressful exposures later in life. As a result, experience-dependent adjustments in developmental structures and neuronal circuitries can elicit vulnerability for stress-related diseases later in life in a stressor- and tissue-related manner (Murgatroyd and Spengler 2011).

A monogenic disease that exemplifies prototypically the need to transit in a synchronized and timely manner through critical periods of neuronal development is presented by Rett syndrome (RTT). Children with RTT develop normally until early childhood whereon they suffer from developmental stagnation (microcephaly, growth retardation, weight loss, and muscle hypotonia) from the age of 6–18 months. As the syndrome progresses further, it manifests stereotypic movements, social withdrawal and unresponsiveness, loss of language, ataxia, seizures, and autonomic perturbations, among other complications (Chahrour and Zoghbi 2007).

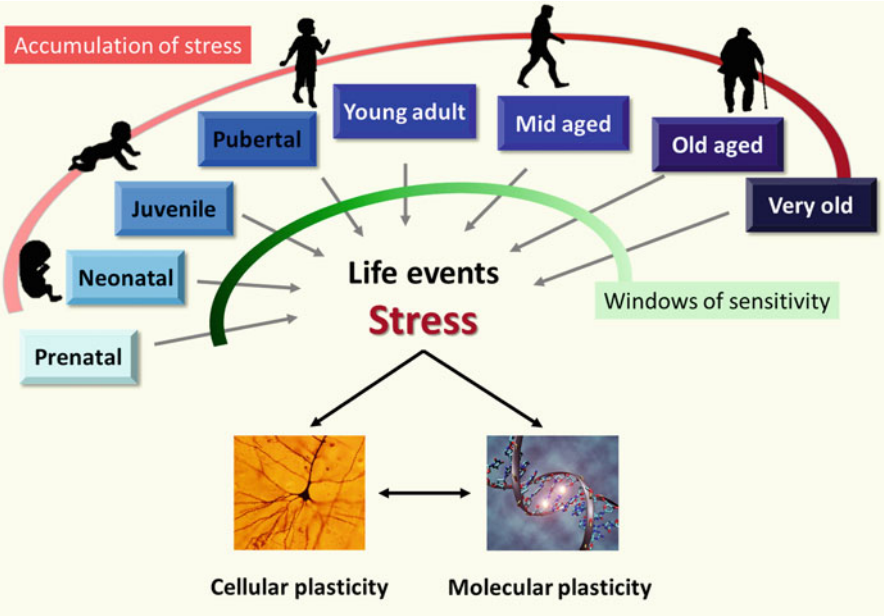

Fig. 5.1 Epigenetic marking across lifespan. Humans pass through different life stages at which experiences can elicit long-lasting changes and adaptations in brain functions comprising cognition, mood, and stress responsivity. Neuronal activity-dependent plasticity enables structural changes and fine-tuning of neuronal circuitries in response to such experiences. Importantly, neuronal activity can also couple to the epigenetic machinery and impose epigenetic marks at genes critical to higher brain functions, behavior, and stress regulation. Both processes, cellular and molecular plasticity, are most prevalent in early life from prenatal stages throughout puberty to decline in late adulthood and aging. On the contrary, the outcomes of stress exposures are thought to accumulate across lifetime. Importantly, adverse early life events can influence long-lasting stress-coping capability and increase the risk for stress-related diseases in later life. Timely therapeutic interventions are looked for to ameliorate, or even reverse, harmful epigenetic marks in response to ELA

Sensory-driven neuronal activity refines cortical circuitries during sensitive postnatal periods of development, and accruing evidence on the molecular and cellular foundations of RTT has prompted the hypothesis of a defect in neuronal plasticity as a result of aberrant synaptic connectivity.

5.2.2 Epigenetic Modifications

All somatic cells of an organism share the same genetic blueprint although they are subject to distinct cell fate decisions during development and adopt vastly differing functions during the process of differentiation and maturation. These changes cannot be attributed to changes in genetic sequence but result from epigenetic mechanisms

that control which genes are switched on and off to produce temporospatial- and cell-specific expression patterns giving rise to various cell identities (Armstrong 2014). So far, the best studied epigenetic mechanisms include DNA methylation and histone modifications that will be briefly summarized here. Apart from these, microRNAs and chromatin remodeling factors are increasingly recognized as important regulatory mechanisms of the epigenetic machinery (Allis et al. 2015).

5.2.2.1 DNA Methylation

DNA methylation refers to the covalent and stable bond of a methyl group (CH_3) to cytosine nucleotides in the DNA (Jeltsch and Jurkowska 2014; Schübeler 2015). In mammals, this modification has been originally thought to occur predominantly at symmetric CpG dinucleotides until the advent of whole genome bisulfite sequencing showed its presence also at non-CpG nucleotides in neuronal, but less in glial, tissues (Lister et al. 2013). While the function of non-CpG methylation remains presently still poorly understood, recent evidence points to a possible role in gene regulation via Mecp2 binding during neuronal maturation (Chen et al. 2015; Gabel et al. 2015).

The transfer of a methyl group onto the cytosine nucleotide is catalyzed by a family of enzymes known as DNA methyltransferases (DNMTs) consisting of DNMT1, DNMT3A, DNMT3B, and DNMT3L (Jeltsch and Jurkowska 2014). DNMT1 recognizes hemi-methylated DNA and is primarily recruited to the replication fork where it maintains methylation marks during replication. DNMT3A and DNMT3B are de novo methyltransferases and are recruited to unmethylated DNA in concert with other DNA- or chromatin-bound factors. DNMT3L itself has no catalytic activity but fulfills a regulatory role by scaffolding enzymatically active members (Kareta et al. 2006).

CpG sites are in general depleted from the genome, apart from interposed stretches of DNA termed CpG islands (CGI) where CpG content stays high. Approximately 70 % of all annotated promoters associate with a CGI and remain in general methylation-free (Saxonov et al. 2006), whereas CpGs outside of CGIs are mostly methylated (Gibney and Nolan 2010). DNA methylation of promoter CGIs has been commonly viewed to associate with gene repression, particularly during cancer development. However, recent studies have shown that the popular view of DNA methylation as an all-purpose repressive mark needs to be revised and applies only to allele-specific methylation of promoter CGIs on the inactive copy of the female X chromosome, to imprinting control regions, and germ cell-expressed genes in somatic cells (Auclair et al. 2014).

In fact, genome-wide methylation analyses revealed that the effects of DNA methylation depend strongly on the underlying sequence, genomic location, and transcriptional status and associate with both gene activation and repression and, possibly, alternative splicing (Weber et al. 2007; Lister et al. 2009; Maunakea et al. 2010; Lister et al. 2013).

Traditionally, DNA methylation has been treated as a rather stable mark with an important role in cell lineage decisions and differentiation, parent-of-origin-dependent

allelic expression of imprinted genes, and the silencing of transposable elements (Hoffmann et al. 2015). Demethylation was originally thought to occur mainly passively during cell proliferation until the transformative discovery of active demethylation (Wu and Zhang 2014). At the core of the demethylation, machinery resides the ten-eleven translocation (Tet) family of enzymes (TET1-3) that catalyze the iterative conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (Tahiliani et al. 2009; Ito et al. 2010) and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al. 2011; Ito et al. 2011). The oxidized residues 5fC and 5caC are recognized by the DNA repair machinery and are then excised from the DNA by the DNA glycosylase TDG (thymine DNA glycosylase). The resulting gap in the nucleotide strand is then repaired by the base excision and/or nucleotide excision repair machinery (BER/NER). Alternatively, AID/APOBEC proteins are proposed to deaminate 5hmC directly to 5-hydroxyuracil (5hmU) followed by the repair of the resulting 5hmU·G mismatch by TDG and SMUG1 (single-strand selective monofunctional uracil DNA glycosylase) (Cortellino et al. 2011).

5.2.2.2 Histone Modifications

Nuclear DNA is tightly wrapped around the four core histones H2A, H2B, H3, and H4 to form nucleosomes that make up the building block of chromatin (Li et al. 2007). With the help of the linker histone H1 and other nonhistone proteins, DNA accessibility is restricted further by compacting the chromatin in higher-order structures (Misteli 2007). On the other hand, the accessibility of chromatinized DNA can be regulated by posttranslational modifications of the free histone tails protruding from the chromatin surface. Selected amino acids are recognized by specific groups of enzymes, which catalyze lysine acetylation (HAT), lysine and arginine methylation (HMT), and serine phosphorylation, among others. These combinatorial modifications promote either a condensed or an open chromatin state and thus regulate the access of the transcriptional machinery to the DNA (Bhaumik et al. 2007). Moreover, specific histone signatures can influence the binding capacities of various proteins of the epigenetic machinery itself such as DNMTs and HATs. Therefore, histone modifications and DNA methylation can reciprocally control each other in deposition and function.

5.3 Mecp2 Protein Structure and Function

5.3.1 Mecp2 DNA Binding and Transcriptional Regulation

Mecp2 is the founding member of the so-called methyl-CpG-binding domain (MBD) proteins (Fig. 5.2a). These proteins share the conserved MBD through which they specifically recognize methylated CpG residues (Hendrich and Bird 1998). The MBD binds symmetrically to methylated CpG dinucleotides, mediated by multiple contacts in the major groove of the DNA double helix in conjunction

with immobilized water molecules (Ho et al. 2008). Further, Mecp2 can additionally bind by means of its A/T-hook motifs to A/T-rich sequences within the minor groove of the DNA. Together, methyl-CpGs and nearby A/T-rich sequences constitute high-affinity Mecp2 DNA binding (Klose et al. 2005).

Once bound to DNA, Mecp2 can exert a variety of functions (Fig. 5.2b). The corepressors Sin3a and histone deacetylases (Hdacs) 1 and 2 are recruited through the central transcription repression domain (Nan et al. 1997; Jones et al. 1998). Alternatively, chromatin compaction can be facilitated by the A/T-hook domains together with α-thalassemia/mental retardation syndrome X-linked protein (ATRX) (Nan et al. 2007). Additionally, interactions with a number of other factors like Dnmt1 (Kimura and Shiota 2003), histone methyltransferase Suv39H1 (Fuks et al. 2003), and the corepressors c-Ski and N-CoR (Kokura et al. 2001) have been reported. Interestingly, Mecp2 binding has also been shown to be associated with unmethylated or hydroxymethylated DNA and non-CpG methylation, further expanding its repertoire of potential regulatory sites (Georgel et al. 2003; Yasui et al. 2007; Kinde et al. 2015; Gabel et al. 2015).

Mutations in *MECP2* are causing 95 % of all RTT cases, develop mostly de novo in the paternal germline, and comprise missense, nonsense, and frameshift mutations (Lyst and Bird 2015). The eight most common point mutations (R106W, R133C, T158M, R168X, R255X, R270X, R294X, R306C) are detected in over 65 % of RTT cases (Fig. 5.2a) (Calfa et al. 2011).

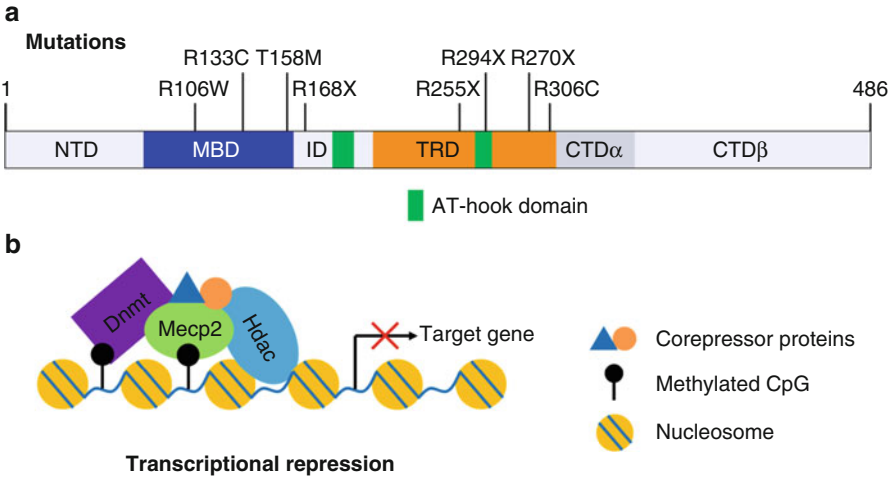

Fig. 5.2 (**a**) MECP2 protein structure. The eight most common mutation sites are shown. *NTD* N-terminal domain, *MBD* methyl-CpG-binding domain, *ID* interdomain, *TRD* transcription repression domain, *CTD* C-terminal domain (total 486 amino acids). (**b**) Mecp2 function. Mecp2 binds to methylated promoters and confers transcriptional repression of target genes through the recruitment of various corepressors. Repression can be reinforced through interactions with histone deacetylases (Hdacs), which catalyze the removal of acetyl groups from free histone tails as well as DNA methyltransferases (Dnmts), which catalyze the transfer of methyl groups onto the DNA

5.3.2 *Mecp2* Target Genes

To gain insight into Mecp2's biological function, potential target genes were inferred from transcriptional profiling of *Mecp2*-null mice (Tudor et al. 2002). Unexpectedly, such analysis displayed only minor perturbations in gene expression arguing against a role as global repressor, as it was originally thought, and favored a more local, and possibly, cell-type specific, role of Mecp2 (Zimmermann et al. 2015). On the other hand, a study conducted by Nuber and coworkers reported upregulation of a number of glucocorticoid target genes including serum glucocorticoid-inducible kinase 1 (*Sgk1*), FK506-binding protein 5 (*Fkbp5*), and *Pomc* in *Mecp2*-null mice across various disease stages (symptom-free, early symptoms, and late symptoms) (Nuber et al. 2005). Interestingly, glucocorticoids, which are known to induce *Sgk1* and *Fkbp5*, were only slightly elevated in Mecp2-deficient mice. In turn, chromatin immunoprecipitation (ChIP) experiments in wild-type mice showed Mecp2 occupancy at regulatory regions of *Sgk1* and *Fkbp5* in the brain as well as for *Pomc* in the pituitary as reported elsewhere (Wu et al. 2014). Thus, in support of a local transcriptional role for Mecp2, repression of these genes can be ascribed to Mecp2 binding rather than increased glucocorticoid levels. An involvement of Mecp2 in gene expression and the stress response was further investigated by McGill and coworkers in a mouse model bearing a truncated *Mecp2* allele ($Mecp2^{308/Y}$). These mice showed enhanced anxiety-like behavior and increased serum corticosterone levels indicative of an abnormal stress response (McGill et al. 2006). In accord with corticotropin-releasing hormone's (CRH) role as a major driver of the HPA axis and associated anxiety, Crh mRNA expression was upregulated in $Mecp2^{308/Y}$ mice brain regions important for the stress response and anxiety (i.e., the paraventricular nucleus of the hypothalamus (PVN), the central amygdala, and the bed nucleus of the stria terminalis). It is important to note that *Crh* expression was still restricted to those neurons where it is normally expressed, indicating for Mecp2 a role in the modulation of gene expression rather than in on-off switches. Interestingly, ChIP experiments indicated Mecp2 binding to the proximal CpG-rich and highly methylated *Crh* promoter only for the wild type but not the truncated $Mecp2^{308/Y}$ protein. Although these findings strengthen the repressive properties of Mecp2 binding at the *Crh* promoter, they have to be reconsidered in the light of a later study (Samaco et al. 2012). Here, duplication of *MECP2*, a genetic condition present in some boys suffering from autism and comorbid anxiety (Ramocki et al. 2009), has been studied in transgenic mice. These animals showed increased anxiety and autism like features in conjunction with increased *Crh* and *Oprm1* (an opioid receptor subtype) expression. Genetic ablation of either *Crh* or its receptor (*Crhr1*) could largely normalize the anxiety phenotype. Taken together, these results indicate an important role of Mecp2 in the regulation of molecular pathways involved in anxiety and social behaviors. On the other hand, the discrepancies between these studies can be explained by a number of experimental limitations. Firstly, the $Mecp2^{308}$ variant lacks key serine residues S421 and S424 that serve as phosphorylation sites important for the regulation of DNA

binding (Zhou et al. 2006). Secondly, none of the investigated transgenic mice models confined the expression of mutated Mecp2 to specific brain regions, and therefore local effects may be confounded by system-wide effects. In this respect, the use of temporospatial knockout models is locked for to advance refined insight into the mode of Mecp2 function at the level of single cells and circuits (Zimmermann et al. 2015).

5.3.3 *Neuronal Activity-Dependent Posttranslational Modifications of Mecp2*

Neuronal plasticity is a cardinal feature of the brain and underlies processes like learning, memory formation, and the capability to adapt to dynamically changing environments. All of these events involve changes in brain structure and neural circuitries in a manner dependent on neuronal activity. Further, neuronal activity can also drive changes in DNA modifications, namely, CpG methylation, and thus provide an additional layer of molecular plasticity. Thereby, cellular and molecular plasticity are likely to influence each other in common biological functions as well as in their development and maintenance (Hoffmann and Spengler 2012).

Currently, Mecp2's involvement in processes underpinning cellular and molecular plasticity is still incompletely understood although several neuronal activity-dependent posttranslational modifications have been identified in the past years that are potentially involved in protein-protein interaction, DNA binding, and transcriptional regulation (Bellini et al. 2014; Ausió et al. 2014; Li and Chang 2014). Among these modifications, a major focus has been placed on phosphorylation with the most carefully studied phosphorylation site being S421. It was firstly observed in 2003 that neuronal activation led to Mecp2 phosphorylation concomitantly with reduced DNA binding (Chen et al. 2003; Martinowich et al. 2003). Interestingly, calcium (Ca^{2+}) influx triggered calcium/calmodulin-dependent protein (CaMKII) kinase-dependent S421 phosphorylation, which was predominantly detected in the brain where it has been also shown to be induced by light exposure in the suprachiasmatic nucleus (SCN) (Zhou et al. 2006). Furthermore, S421 phosphorylation was reported in specific hypothalamic paraventricular neurons following exposure to early life stress (ELS) (Murgatroyd et al. 2009). Alternatively, Mecp2 S421 phosphorylation can also be triggered by activation of dopamine or serotonin receptors in the nucleus accumbens (Hutchinson et al. 2012a). Similarly, an increase in extracellular monoamine neurotransmitter concentrations following antidepressant treatments such as imipramine was also reported to selectively induce S421 phosphorylation in GABAergic interneurons (Hutchinson et al. 2012b).

Functionally, S421 phosphorylation is thought to reduce Mecp2's binding capacity to the methylated *Bdnf* promoter and to enhance neuronal

activity-dependent transcription and subsequent release of this neurotrophic factor (Chen et al. 2003). Further, S421 phosphorylation is critical to Mecp2-dependent regulation of dendritic growth and spine maturation and suggests that this modification mediates neuronal activity-driven changes in distinct gene expression programs (Zhou et al. 2006). Analyses of *Mecp2*$^{S421A/y}$ mice, in which Mecp2's phosphorylation site at S421 has been eliminated by replacement with alanine, revealed only a mild phenotype with no differences in anxiety-like behavior and locomotion. At the same time, increased dendritic complexity and inhibitory neurotransmission were accompanied by deficits in hippocampal learning and memory. Mecp2 phosphorylation at S421 has been also detected in conjunction with phosphorylation of nearby S424, and simultaneous modification of either residue has been suggested to be critical to Mecp2's functions (Li et al. 2011). In support of this view, *Mecp2*$^{S421A;S424A}$ knock-in mice manifest a phenotype opposed to *Mecp2*$^{S421A/y}$ characterized by an increased performance in hippocampus-dependent spatial learning/memory tasks and expression changes in genes underlying these functions. Additionally, Mecp2$^{S421A;S424A}$ bound more tightly to the *Bdnf* promoter when compared to the wild-type protein and conferred increased *Bdnf* transcription in the hippocampus (Li et al. 2011).

In this context, it is important to note that recent studies suggest that phosphorylation at several residues unrelated to S421/S424 may serve coregulation of DNA binding as well as the recruitment of various coregulators once Mecp2 is bound to the DNA (Gonzales et al. 2012; Ebert et al. 2013). Hence, the complex, and possibly combinatorial, use of these phosphorylation sites may integrate Mecp2's overall functions, and future studies are looked for to elucidate the interplay between phosphorylation sites controlling DNA binding versus those controlling coregulator recruitment.

In support of this scenario, a second important phosphorylation site controlling DNA binding seems to be Serine-80 (S80). In contrast to S421, this site is dephosphorylated by neuronal activity, facilitates the association of Mecp2 with chromatin, and has also been detected in non-neural cells. Replacement of S80 with alanine in *Mecp2*^{S80A} knock-in mice leads to locomotor deficits and reduced Mecp2 occupancy at several target genes, including *Pomc* (Tao et al. 2009). S80 phosphorylation is catalyzed by homeodomain-interacting protein kinase 2 (HIPK2) and might be functionally involved in the induction of apoptosis during neurodevelopment (Bracaglia et al. 2009).

In summary, the molecular mode of Mecp2's action is still subject to current investigations. Mecp2 has been described as transcriptional activator as well as repressor both on a single gene and genome-wide level. This may be due to variable functions depending on the interaction partner, sequence context, chromatin environment, and different posttranslational modifications. All in all, Mecp2 seems to participate in various cellular functions in a rather context-dependent than an all-purpose manner with an emphasis on fine-tuning target gene expression. This reservation has to be taken into account when studying Mecp2's functions in highly heterogeneous tissues like the brain.

5.4 Early Life Stress and the HPA Axis

ELA, as defined by the National Comorbidity Survey Replication Study (Green et al. 2010), comprises a variety of conditions consisting of interpersonal loss (parental divorce or death and other separation from parents or caregivers), parental maladjustment (criminality, violence, substance abuse, and mental illness), maltreatment (physical or sexual abuse and neglect), life-threatening childhood physical illness in the respondent, and extreme childhood family economic discrimination. In 2012, maltreatment rates corresponding to 9.1 per 1000 children were reported for the US population with neglect representing the most common form of child maltreatment (79.5 %) (U.S. Department of Health and Human Services et al. 2013).

The tight reciprocal relationship between mother and infant during early life plays a fundamental role in shaping the child's psychological, cognitive, and affective development. Importantly, this also includes the integrated response to stressful events in a protective parental environment (Shonkoff et al. 2000; Myers and American Professional Society on the Abuse of Children 2011). If infants lack maternal care and attention and are raised in an adverse environment deprived of positive mental and emotional experiences, they fail to reach developmental milestones. Neglected toddlers show less eye contact and less smiling; they are irritable and become increasingly withdrawn, less responsive, and less engaged (England and Sim 2009).

Adverse early life experiences increase the risk for major depression by fourfold (Felitti et al. 1998). Moreover, a strong dose-response relationship between childhood adversities and mental health in adulthood together with a strong impact of the severity of adverse childhood experiences on recurrent depression and suicidality could be shown (Dube et al. 2001; Edwards et al. 2003; Chapman et al. 2004). In brief, ELA is a well-characterized risk factor for the vulnerability to depressive diseases and the severity of depressions' clinical course and progression.

5.4.1 The HPA Axis

A broad variety of physiological and psychological stressors are integrated by activation of the HPA axis as the major mediator of the stress response, which in turn serves to coordinate the cognitive, behavioral, and metabolic responses of the exposed organism. Perception of extrinsic and intrinsic stressors triggers the release of two hypothalamic neuropeptides, CRH and AVP from the parvocellular neurons of the PVN. Upon binding to their respective receptors at the anterior pituitary, POMC mRNA is produced and translated into a precursor protein that is posttranslationally processed to adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal cortex to produce and secrete cortisol (in humans) and corticosterone (in humans, rats, and mice). Corticosteroids act through ligand-gated transcription

factors, namely, the nuclear glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). GR activation in the PVN and anterior pituitary shuts down the stress response via negative feedback mechanisms and thus contributes to reinstate the homeostatic equilibrium (de Kloet et al. 2005).

5.4.2 *Mecp2 Mediates Early Life Stress on Avp Expression*

A well-established model for inducing ELS in rodents is maternal separation during which mice pups are separated in the first 10–14 days of their life for 3 h daily from the dam (Pryce and Feldon 2003; Millstein and Holmes 2007). We could previously show that ELS in C57BL/6 mice elicits enduring changes in the responsiveness of the HPA axis. Long-lasting hyperactivity of the HPA axis in the offspring was evidenced by corticosterone hypersecretion during the resting state and an increased response after exposure to an acute stressor and further associated with behavioral changes comprised of memory deficits and increased immobility in the forced swim test (Murgatroyd et al. 2009). This phenotype concurred with increased *Avp*, but not *Crh*, expression in the PVN. It is important to note that changes in *Avp* expression were confined to the PVN, but absent in the supraoptic nucleus (SON), with a role in the stress response and fluid homeostasis, respectively. The upregulation begins as early as 10 days, directly after termination of the stressor, and persists at least until 1 year (Fig. 5.3). In order to investigate the molecular basis of sustained *Avp* expression, bisulfite sequencing was applied and showed a robust hypomethylation at specific CpG residues in the downstream enhancer region at 6 weeks that declined until 1 year. These differentially methylated, ELS-responsive CpG sites localized to a cluster of potential high-affinity DNA-binding sites for Mecp2 (Klose et al. 2005). In fact, Mecp2 binding was reduced in ELS-exposed mice from 6 weeks onward. Moreover, Mecp2 binding was also diminished at 10 days (P10); even so, *Avp* enhancer methylation was undistinguishable between treatment groups (Fig. 5.3). Therefore, we hypothesized that ELS-dependent neuronal activity triggers Mecp2 phosphorylation and, in turn, reduced DNA-binding capacities. In support of this view, at P10, increases in Mecp2 S421 phosphorylation were detected jointly with calmodulin kinase II phospho-immunoreactivity in Avp-expressing paraventricular neurons. The phosphorylated and therefore active form of calmodulin kinase II has been suggested to couple neuronal activity-driven Ca^{2+} influx with Mecp2 phosphorylation (Zhou et al. 2006; Tao et al. 2009). Consistent with this concept, Mecp2 S421 phospho-immunoreactivity was only enhanced shortly after termination of the maternal separation.

In summary, these findings support the hypothesis that Mecp2 dissociation initially derives from neuronal activity-dependent phosphorylation that triggers acute derepression of *Avp*. Following on, reduced Mecp2 binding facilitates *Avp* enhancer hypomethylation that becomes the driving force for sustained overexpression (Fig. 5.3). Hereby, Mecp2's protective role against *Avp* enhancer demethylation may also take place in the context of the maturating postnatal brain methylome (Lister et al. 2013).

A final question concerns the transcriptional outcome of Mecp2 binding at the *Avp* enhancer region. While some studies suggested an activating role for DNA-bound

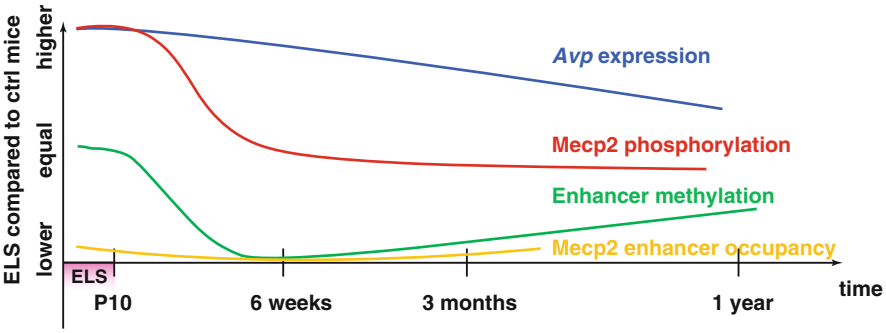

Fig. 5.3 Model for epigenetic marking of *Avp* over lifetime. Regulation of Avp mRNA expression in ELS-exposed mice when compared to controls (ctrl). In ELS mice, *Avp* expression is increased for up to 1 year compared to controls through serially acting mechanisms. Immediately after stress, at postnatal day 10 (P10), Mecp2 phosphorylation is increased by neuronal activity-induced calcium influx leading to the activation of calcium-calmodulin-dependent protein kinases. Mecp2 phosphorylation facilitates disassociation from the *Avp* enhancer region and a reduced recruitment of DNA methyltransferases and histone deacetylases. Consequently, *Avp* enhancer methylation is lowered and underpins reduced Mecp2 binding. Thus, adverse early life experiences have become hardcoded and translate into increased Avp expression and stress susceptibility. At later time points, differences in *Avp* expression and enhancer methylation decline but persist at least until 1 year

Mecp2, both locally and genome-wide (Chahrour et al. 2008; Samaco et al. 2012), other studies suggest an inhibitory role (Chen et al. 2003; Martinowich et al. 2003; Nuber et al. 2005; McGill et al. 2006; Skene et al. 2010; Guy et al. 2011). In view of *Avp* derepression upon Mecp2 dissociation, a repressive role would be anticipated at the enhancer region. Consistent with this hypothesis, Mecp2 was found to be associated with repressive histone marks at the *Avp* enhancer as evidenced by sequential ChIP experiments (Murgatroyd and Spengler 2014). Further, Mecp2 binding favored the recruitment of de novo Dnmts (Dnmt3a and Dnmt3b) and provides a salient explanation for the decrease in methylation upon loss of Mecp2 binding during postnatal global reconfiguration of the brain methylome (Lister et al. 2013).

Overall, ELS, as a result of maternal separation, leaves an enduring epigenetic mark at the *Avp* enhancer that influences *Avp* expression throughout a lifetime. First, a molecular memory trace is formed through activity-dependent phosphorylation of Mecp2 and subsequently reduced recruitment of Dnmts to the *Avp* enhancer region during postnatal brain development. Once established DNA hypomethylation accounts for reduced Mecp2 binding and preserves the molecular memory trace even in the absence of the initial event.

5.4.3 Mecp2 Mediates Early Life Stress on Pomc Expression

The joint action of Crh and Avp at the pituitary induces the expression of *Pomc* and the secretion of its posttranslational product ACTH. Consistent with higher Avp levels in early life stressed mice, Pomc levels were as well elevated under resting

conditions and following application of the AVP/CRH challenge test (Wu et al. 2014). These expression differences were associated with hypomethylation of multiple CpG residues at the proximal *Pomc* promoter throughout lifetime whereby the most ELS-responsive CpG residues had previously been shown to be critical for *Pomc* expression. The functional role of DNA methylation at these CpG residues was further assessed in transfection assays with patch-methylated *Pomc* promoter constructs that evidenced efficient repression by Mecp2 but not by other MBD proteins (Wu et al. 2014). Consistent with these findings, several potential high-affinity Mecp2 binding sites in the ELS-responsive region were verified by electrophoretic mobility shift assays in conjunction with mutational analysis. Moreover, in vivo ChIP experiments indicated that Mecp2 occupancy at the proximal *Pomc* promoter associated with the recruitment of Dnmt1 and Hdac2 (Wu et al. 2014). Interestingly, Mecp2 binding to as well as the presence of Dnmt1 and Hdac2 at the proximal *Pomc* promoter was reduced in ELS-exposed mice concomitantly to increased *Pomc* expression.

In summary, these results suggest that Mecp2 is also mediating the impact of adverse early life experiences on pituitary Pomc expression through the induction of DNA hypomethylation at key regulatory regions.

5.5 Conclusion

While the regulatory role of epigenetic modifications has been firmly established over the past two decades and still evolves, the role of multilayered gene-environment interactions in shaping the social epigenome has been only recently recognized. As a rather robust, albeit dynamic mark, DNA methylation has received considerable attention due to its capability to inscribe various experiences on the long term into the epigenome. Adverse early life experiences can interact with the epigenome of developing organisms, including the brain, and thus leave long-lasting, or even persistent, memory traces across lifespan. Such hardcoded records of early life experiences may help an organism to prepare for and adapt to current and future demands imposed by an ever-changing environment. Although of potential benefit, experience-dependent epigenetic marking can come at a cost when early responses do not match the later environment and turn out to increase the vulnerability for certain environmental challenges that come up in adulthood (Gluckman et al. 2009; Bateson et al. 2014; Hoffmann and Spengler 2014; Patchev et al. 2014).

ELA is increasingly recognized as an important risk factor for the development of stress-related disorders, a more severe course, and poor treatment responses. Sustained upregulation of key drivers of the stress response, like hypothalamic *Avp* and pituitary *Pomc*, is caused through experience-dependent epigenetic marking in mice. ELS reduces DNA methylation at critical regulatory sequences in the *Avp* enhancer and proximal *Pomc* promoter regions. Hereby, the role of Mecp2 as important "reader and writer" of DNA methylation in response to ELS needs to be emphasized and substantially extends its original role in neuronal plasticity and as the

genetic cause of the neurodevelopmental disorder RTT (Zimmermann et al. 2015). Mecp2 is not only involved in the recognition of epigenetic marks but also translates these into the recruitment of protein complexes conferring repressive histone modifications and DNA methylation, and both functions seem to be sensitive to adverse early experiences. Functionally, neuronal activity-driven phosphorylation of Mecp2 S421 seems to be necessary to bridge early social experiences to the regulation of DNA binding, deposition of repressive histone marks, and maintenance of DNA methylation on the long run.

Overall, the incidence of ELA and its role for depressive disorders is on the rise. One important factor in transducing adverse early experiences through epigenetic marking into adulthood can be assigned to Mecp2. To advance future treatments in human, not only an understanding of how adverse early experiences are hardcoded onto the DNA but also possible mechanisms to ameliorate, or even reverse, such unfavorable epigenetic changes are looked for.

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Chapter 6
Epigenetic Programming of Hypothalamic Pomc Regulates Feeding and Obesity

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Abstract The environment can have a long-lasting influence on an individual's physiology and behavior. While some environmental conditions can be beneficial and result in adaptive responses, others can lead to pathological behaviors (Franklin et al. Neuron 75:747–761, 2012). The period of perinatal development is one of the most critical windows during which adverse conditions can influence the growth and development of the fetus, as well as the offspring's postnatal health and behavior (Franklin et al. Neuron 75:747–761, 2012). Moreover, recent evidence points to the possibility that changes which occur in the individual can sometimes pass between generations even if the offspring are not directly exposed to the stimulus (Gapp et al. Biology 36:491–502, 2014). Epigenetic alterations are prime candidates for the major molecular mechanism acting at the interface between genetic and environmental factors. Different studies showed that environmental factors, such as fetal alcohol exposure, maternal stress, under or overnutrition, or smoking exposure during sensitive periods affect gene expression in the offspring via altering epigenetic mechanisms, sometimes even across multiple generations (Begum et al. Endocrinology 154:4560–4569, 2013; Blaze and Roth Int J Dev Neurosci 31:804–810, 2013; Laufer et al. Dis Model Mech 6:977–992, 2013; Novakovic et al. Epigenetics 9,

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2013). In this review, we discuss the involvement of the proopiomelanocortin (POMC) system, one of the most important regulators of energy balance, and describe how epigenetic changes such as histone modifications and DNA methylation modulate *Pomc* gene expression and function. We also summarize the recent findings from animal models which show that both diet-induced obesity (DIO) and malnutrition program the POMC system of subsequent generations via epigenetic mechanisms.

Keywords Proopiomelanocortin (POMC) • Obesity • Leptin • High-fat diet • Methylation

6.1 Obesity Epidemiology and Its Consequences

Obesity is a growing public health concern, which has reached epidemic proportions in the last few decades. By 2014 the prevalence of obesity doubled itself, reaching to more than one third of the adult population (35.7 %) and almost 17 % of youth population (Ogden et al. 2007). According to the 2013 OECD reports, the majority of the population, and one in five children, are overweight or obese in the OECD area. A nearly tenfold variation in rates of obesity and overweight is observed across OECD countries. Sustained obesity has profound consequences in one's life, from psychological symptoms to serious comorbidities that may markedly diminish both the quality and length of life (Ogden et al. 2012), and it is regarded by the World Health Organization as a chronic disease that constitutes a risk factor for hypertension, cancer, and many additional diseases (Walley et al. 2006).

Obesity results from a wide spectrum of genetic and environmental factors that lead to imbalance between energy intake and energy expenditure (Schwartz et al. 2000). Increased intake (hyperphagia), diets rich in fats and sugars, and reduced physical activity are the environmental factors implicated in the prevalence of obesity in Western societies (Schrauwen and Westerterp 2000). Over the past decades, research has identified signaling molecules that are critical for normal energy homeostasis (Bouret and Simerly 2006; Martin-Gronert and Ozanne 2013; Schwartz et al. 2000). These affect highly complex processes including the digestive system, liver, adipose tissue, and neuroendocrine feedback loops (Cone 2005; Marchesini et al. 2008; Weller 2006). There is also evidence from genetic epidemiology suggesting that genetic factors are involved in determining the susceptibility to gaining or losing fat in response to certain diets (Barsh and Schwartz 2002). Despite the evidence that genetic factors play a central role in the development of obesity and the increasing number of genes identified, little is known about the regulation of these genes in response to alterations in the environment (Begum et al. 2012; Plagemann et al. 2009).

6.2 Regulation of Energy Homeostasis

Intake and energy expenditure must be regulated in order to achieve energy homeostasis. The major determinant of meal size is the onset of satiety, which is a biological state induced by neurohumoral stimuli generated during food intake that leads to meal termination (Smith 2000). Different signals involved in energy homeostasis may control food intake primarily by adjusting the size of individual meals (Schwartz et al. 2000). Positive feedback is initiated by the taste, sight, and smell of food, and its main function is to draw the organism to food and sustain the duration of the meal, enlarging in this manner the meal size (Smith 2000). Previous research suggested that neurotransmitters such as dopamine and norepinephrine mediate the positive feedback signals. Conversely, negative (satiety-inducing) signals, such as cholecystokinin (CCK), that contact the hindbrain are initiated by mechanical or chemical stimulation of the stomach and small intestine during food ingestion (Weller 2006).

In contrast to the immediate response to a meal signal, there are long-term satiety signals. Under normal conditions, brain regions involved in long-term energy balance, by definition, must sense the amount of existing fuel in the body and use this knowledge to adjust energy intake and expenditure (Schneeberger et al. 2014). However, body energy levels are not sensed via circulating levels of fuel molecules (i.e., glucose or lipids) under most circumstances, but via signals such as leptin and insulin which index the amount of energy (fat) in store and the availability of glucose, respectively (Fan et al. 2011). When leptin or insulin levels in the circulation are high, neurons in the brain interpret a high energy status and thus inhibit food intake and increase energy expenditure (Tups 2009).

6.3 The Regulation of Energy Balance in the Hypothalamus

One of the most important regions in the brain that mediate long-term signals is located in the ventral diencephalons, known as hypothalamus. The hypothalamus regulates a variety of systems and functions necessary for our body, such as eating behavior, energy and salt balance, thermoregulation, stress responses, growth, and control of gonadal activity. In the hypothalamus, primary importance has been given to the arcuate nucleus (ARC) (Bouret and Simerly 2006). The ARC is located in the mediobasal hypothalamus. It contains appetite suppressor (anorexigenic) and appetite stimulator (orexigenic) populations of neurons which act as first-order neurons in the hypothalamic response to circulating peripheral hormones such as insulin, leptin, and ghrelin (Bouret and Simerly 2006). Mediated by hypothalamic leptin receptors (LEPR) and insulin receptors (IR), decreased levels of leptin and insulin physiologically lead to upregulation of the hypothalamic expression of orexigenic neuropeptides and consecutively to hyperphagia and increased body weight and

vice versa (Cone 2005). The orexigenic neuropeptides include neuropeptide Y (NPY) and agouti-related protein (AgRP). NPY is expressed predominantly within the ARC and transported to second-order hypothalamic sites such as the paraventricular (PVN) and dorsomedial (DMH) nuclei where NPY receptors mediate the physiological role (Cone 2005). AgRP is a potent antagonist of melanocortin receptors (also located in the PVN) and opposes their anorexigenic action. AgRP neurons also inhibit anorexigenic neuronal activity by releasing inhibitory gamma-aminobutyric acid (GABA) (Cowley et al. 2001). Genetic ablation of AgRP neurons in adult mice leads to starvation (Gropp et al. 2005). As a counter-regulation, anorexigenic neuropeptides, the posttranscriptional cleavage product of POMC, alpha-melanocyte-stimulating hormone (alpha-MSH), and cocaine- and amphetamine-regulated transcript (CART) are expressed in the ARC, decreasing food intake and body weight (Cone 2005).

Downstream of the arcuate nucleus, neurons in hypothalamic areas including the PVN, zona incerta, perifornical area (PFA), and lateral hypothalamic area (LHA) are richly supplied by axons from Npy/AgRP and Pomc/CART neurons (Schwartz et al. 2000). These second-order neurons are stimulated by melanocortin and/or CART signaling, but are inhibited by NPY signaling. Second-order neurons include those that express thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and oxytocin (OXY) in the PVN (which cause anorexia), and neurons that express orexins and melanin-concentrating hormone (MCH) in the PFA and LHA (which increase feeding) (Cone 2005; Schwartz et al. 2000 (Fig. 6.1).)

6.3.1 Leptin Action in the Hypothalamus

Leptin, a polypeptide hormone that is produced by adipocytes in proportion to their size and number, links changes in body stores to adaptive responses in the central control of energy balance (Myers et al. 2010). By binding to and activating of its receptor (LEPR) in the brain, leptin decreases food intake while increasing energy expenditure (Myers et al. 2010). The *Lepr* gene produces multiple leptin receptor isoforms (a, b, c, d, e, and f) via alternative mRNA splicing (Bjorbaek et al. 1998). All isoforms have an extracellular leptin-binding domain, but only the longest form, LEPRb, contains a full-length intracellular domain required for cell signaling (Friedman and Halaas 1998). Genetic deficiency of LEPRb results in morbid obesity in animals, indicating that LEPRb is required for leptin action (Morris and Rui 2009; Pan et al. 2014).

Leptin levels regulate the expression levels of neuropeptides by a signal transduction mechanism. Upon leptin binding, the extracellular domain of the LEPRb undergoes a conformational change to activate its associated janus kinase 2 (Jak2) tyrosine kinase (Bates et al. 2003). Activated Jak2 promotes the tyrosine phosphorylation of several intracellular residues on LEPRb (also on Jak2 itself), and each tyrosine phosphorylation site recruits a specific set of downstream molecules to promote specific intracellular signals (Myers et al. 2010). LEPRb contains three

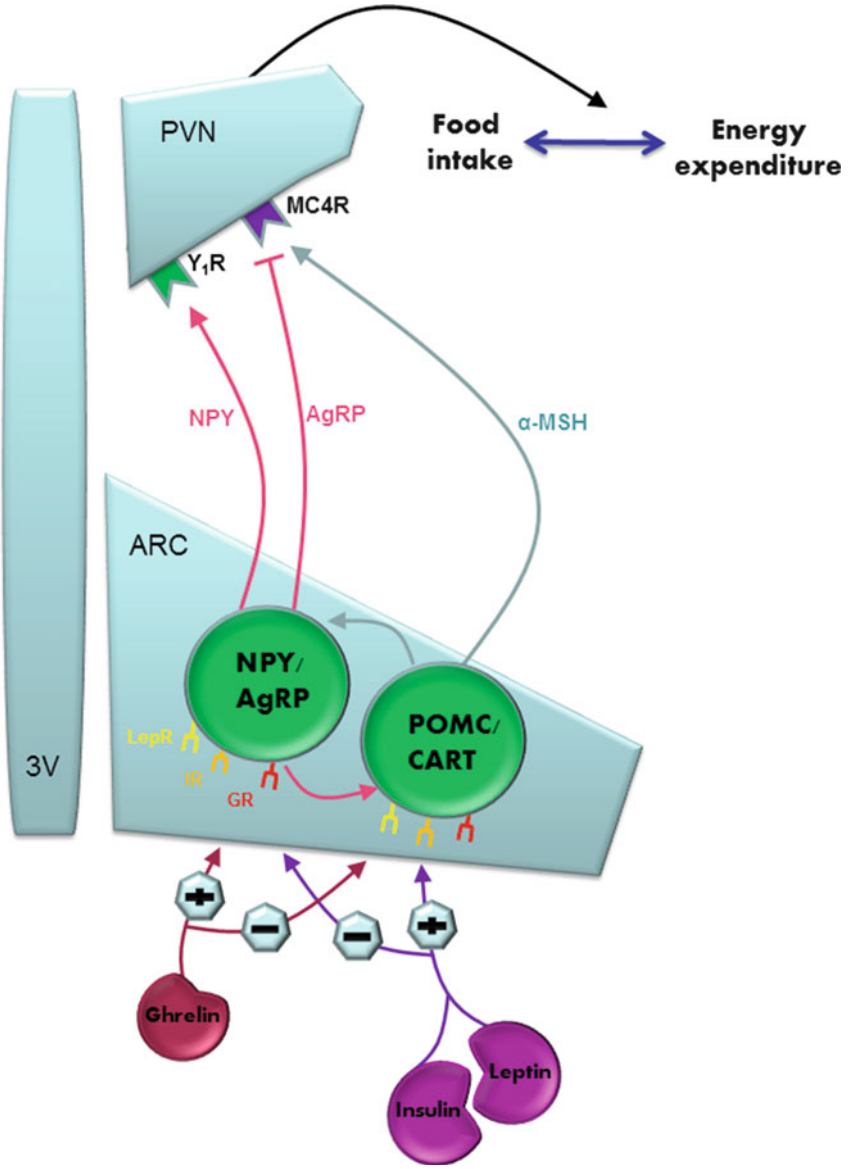

Fig. 6.1 Control of energy homeostasis by arcuate nucleus neurons: There are two sets of neurons in the arcuate nucleus that are regulated by circulating hormones—AgRP/NPY that stimulate food intake and decrease energy expenditure and POMC/CART neurons that inhibit food intake and increase energy expenditure. Insulin and leptin are hormones that circulate in proportion to body adipose stores; they inhibit AgRP/NPY neurons and stimulate adjacent POMC/CART neurons. Ghrelin is a circulating peptide secreted from the stomach that can activate AgRP/NPY neurons, thereby stimulating food intake. *LepR* leptin receptor, *IR* insulin receptor, *GR* ghrelin receptor, *MC4R* melanocortin receptor 4, Y_1R neuropeptide Y receptor Y1

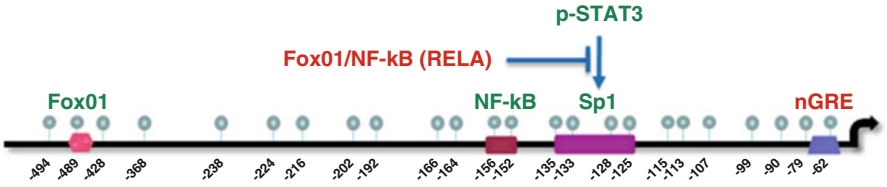

Fig. 6.2 Sequence map of the proopiomelanocortin (*Pomc*) gene promoter region including CpG dinucleotides (lollipop) and functionally regulatory elements (boxed) that are critically involved in the transcription of *Pomc*. Leptin binding to LepR, activating STAT3 by phosphorylation. p-STAT3 translocates into the nucleus and activates POMC promoter activity through its interaction with SP1–POMC promoter complex. FoxO1 and NF-κB can bind to p-STAT3 in the nucleus, preventing STAT3 from interacting with the SP1–POMC promoter complex and, consequently, inhibiting STAT3-mediated leptin activation of POMC promoter. In addition, FoxO1 and NF-κB are also independently able to bind to the POMC promoter and to promote its transcription. Positive signals (*green*): signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), specificity protein 1 (Sp1), and forkhead box O1 (FoxO1). Negative signals (*red*): negative glucocorticoid response element (nGRE) and FoxO1

distinct tyrosine phosphorylation sites: Tyr985, Tyr1077, and Tyr1138 (Gong et al. 2007). Tyr1138 recruits signal transducer and activator of transcription (STAT) 3, a latent transcription factor, which subsequently becomes tyrosine phosphorylated (pSTAT3) by Jak2, enabling its nuclear translocation and promoting its transcriptional effects. Similarly, Tyr1077 recruits and mediates the phosphorylation and activation of a related transcription factor, STAT5 (Gong et al. 2007). Tyr985 recruits the tyrosine phosphatase PTPN11 (protein tyrosine phosphatase, non-receptor type 11), also called SHP2, which controls extracellular signal-regulated kinase (ERK) activation (Bates et al. 2003).

6.3.2 Pomc Gene: Structure and Function

In rat, the *Pomc* gene, localized at chromosome 6, contains 3 exons, 2 introns, and a promoter region of approximately 500 base pairs. The rat *Pomc* gene promoter is located in a CpG island (CGI) containing 24 CpG residues. This CGI comprises DNA binding sequences of various regulatory elements that were characterized to be relevant for transcriptional regulation of the *Pomc* gene (Plagemann et al. 2009). This included the consensus sequences of specificity protein 1 (Sp1), signal transducer and activator of transcription 3 (STAT 3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and more (Plagemann et al. 2009; Shi et al. 2013; Zhang et al. 2014). In addition, other transcription factors such as negative glucocorticoid receptor element (nGRE) and forkhead box O1 (FoxO1) were found to negatively regulate *Pomc* expression. Sequence analysis of this region found apparent sequence conservation for the NF-κB and STAT3–SP1-binding sites and the same spatial arrangement of the two binding sites in several species across

mammals (Shi et al. 2013), suggesting that this may be an important architectural feature (Fig. 6.2).

The *Pomc* gene is predominantly expressed in the pituitary and in the hypothalamus (Autelitano et al. 1989). *Pomc* mRNA is also detected in the amygdala, cortex, testes, lymphoid cells, adrenal medulla, ovaries, placenta, and some tumor tissues (Autelitano et al. 1989). The size of the mature *Pomc* transcript is about 1200 nucleotides in the pituitary and the hypothalamus. (Jeannotte et al. 1987). The *Pomc* gene is translated into a 241-amino-acid precursor polypeptide, which is cleaved in a tissue specific fashion by prohormone convertases 1 and 2 to yield a variety of bioactive peptides (Castro and Morrison 1997). There are eight potential cleavage sites within the *Pomc* precursor, and different tissues contain specific convertases to produce a variety of peptides that include α-MSH, β-endorphin, β-lipotropin (β-LPH), and adrenocorticotropic hormone (ACTH) (Autelitano et al. 1989; Castro and Morrison 1997). Pomc-derived peptides play diverse roles in pathophysiology, including obesity, depression, skin pigmentation, adrenal development, and regulation of the hypothalamic–pituitary–adrenal (HPA) axis. In the hypothalamus, placenta, and epithelium, all eight potential cleavage sites may be used to produce peptides responsible for energy homeostasis, pain, perception, melanocyte stimulation, and immune responses (Cone 2005; Cone et al. 1996; Mountjoy et al. 1994).

6.3.3 The Leptin/Pomc Pathway

Mediated by leptin/insulin signaling, the expression of hypothalamic Pomc is facilitated by a variety of signal transduction pathways (Bouret and Simerly 2006; Cowley et al. 2001). Among those pathways, disruption to the STAT3 binding site in LEPRb, or deletion of neuronal STAT3, results in the most meaningful decrease in hypothalamic Pomc expression, severe hyperphagia, and morbid obesity (Fan et al. 2011). This indicates that the LEPRb/JAK2/STAT3 pathway in the brain is required for the antiobesity actions of leptin (Bates et al. 2003). As described above (1.3.2), leptin binds to LEPRb, leading to phosphorylation and nuclear translocation of STAT3 (Bates et al. 2003). Activated STAT3 can potentially bind to the trans-acting transcription factor 1 (SP1)–*Pomc* promoter complex to activate Pomc transcription (Yang et al. 2009). The expression of Sp1 is physiologically stimulated by insulin, and it has been shown that Sp1 binding to the *Pomc* promoter is essential for the antiobesity action of leptin (Pan et al. 2001; Yang et al. 2009). After transcription, the cleaved product of POMC–α-MSH is axonally transported to the PVN, where it binds to the G protein-coupled melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R) (Mountjoy et al. 1994), activating second-order neurons to mediate satiety and increase energy expenditure. It was found that deletion of either MC3R or MC4R results in severe obesity in mice, indicating the pivotal role of these receptors in energy homeostasis systems (Cone et al. 1996).

6.4 Epigenetics

The term "epigenetics" was first introduced by the developmental biologist Conrad Waddington in 1940 to describe the gene–environment interactions that result in specific phenotypes (Goldberg et al. 2007). While Waddington originally used this concept in a developmental context, the term's current use has extended to describe the study of heritable changes (both mitotic and meiotic) in gene expression that are not due to changes in DNA sequence (Goldberg et al. 2007). The concept of epigenetics was recently revised by the British scientist Adrian Bird who proposed that epigenetic events are the structural adaptation of chromosomal regions (genes) to register, signal, or perpetuate altered activity states (Bird 2007). In this new definition of *epigenetics*, both chromatin marks and DNA modifications, regardless of their heritability, are considered as epigenetic events.

One of the primary functions of epigenetic processes is to remodel chromatin and thereby activate or silence genes. Chromatin comprises the DNA helix, which wraps around octamers of histone proteins to form nucleosomes (Luger et al. 1997). It can be structurally remodeled by covalent modification of the DNA and histones, in particular by DNA methylation (5mC), and by posttranslational histone modifications (PTHM) (Day and Sweatt 2012). In addition to 5mC and PTHM, increasing evidence has pointed to the importance of non-coding RNAs (ncRNAs) as an additional means of gene regulation. ncRNAs exist in a diverse range of sizes, and unlike messenger RNA (mRNA), they are not translated into proteins but can induce mRNA degradation or act as guides of components of the epigenetic machinery to specific DNA sequences (Ghildiyal and Zamore 2009).

6.4.1 DNA Methylation

DNA methylation is a covalent chemical modification involving the addition of a methyl group to the fifth position of the pyrimidine ring in the nucleotide cytosine and results in the generation of 5-methylcytosine (5mC) (Miranda and Jones 2007). In mammals, DNA methylation mostly occurs in the context of CpG dinucleotides, which consist of a cytosine linked via a phosphate group to guanine (Miranda and Jones 2007). Genome regions enriched with CpG dinucleotides are known as CpG islands and usually locate at promoter regions of approximately 70 % of all annotated genes. 5mC modification at CpG dinucleotides is a widespread modification known to be associated with gene silencing. However, recent research revealed that 5mC can also appear in a non-CpG manner, although the function of this is still not very clear (Barres et al. 2009; Schubeler 2015).

One model suggests that 5mC can directly impede the binding of transcriptional factors to their target sites, thus prohibiting transcription (Miranda and Jones 2007). Many transcription factors are targeted to CG-containing sequences, and methylation of CpG sites within these sequences has been shown to prevent the binding of

these proteins to these sites (Day and Sweatt 2012). For example, it was found that methylated DNA sequences interfere with the binding of major transcription factors such as E2F or cAMP response element-binding protein (CREB) (Campanero et al. 2000; Day and Sweatt 2012) which mediate a variety of cellular functions. While the consequence of 5mC is generally thought of as transcriptional silencing, methylation of repressor protein-binding sites can lead to increased gene expression. For example, the imprinted gene insulin-like growth factor 2 (Igf2) can be activated if the upstream repressor sites are differentially methylated in the paternal allele (Eden et al. 2001). Most of the other proposed mechanisms are based on the idea that methylation of CpG sequences can alter chromatin structure by effecting histone modifications through the recruitment of methyl-CpG-binding proteins that can serve as a platform for the assembly of histone deacetylases and histone methyltransferases among other repressor complexes.

In mammalian cells, DNA methylation is catalyzed by DNA methyltransferases (DNMTs) that use S-adenosyl methionine (SAM) as the methyl donor (Szyf and Bick 2013). There are four known DNMTs (DNMT1, 2, 3a and b) which can be subcategorized into two groups by their functions: de novo and maintenance methylation. DNMT3a and DNMT3b catalyze de novo methylation, are essential for normal development, and share at least partially overlapping functions in the establishment of cellular DNA methylation patterns (Miranda and Jones 2007). Maintenance DNA methylation activity is essential for preserving DNA methylation patterns in each cellular DNA replication cycle. DNMT1 functions as a maintenance DNA methyltransferase and specifically recognizes hemi-methylated CpGs following DNA replication of the daughter strand (Mohan and Chaillet 2013). A fourth enzyme previously known as DNMT2 is not a DNA methyltransferase. However, research showed that human DNMT2 can work as a tRNA methyltransferase which specifically methylates cytosine 38 in the anticodon loop (Goll et al. 2006). It is not known how many RNA species can be methylated by Dnmt2; thus the functions of DNMT2 still need to be determined in the future.

5mC was until recently the only nucleotide variant studied in terms of epigenetics in eukaryotes. However, 5mC has turned out to be just one component of a dynamic DNA epigenetic regulatory network, as three additional cytosine variants were identified in the mammalian genome. In 2009, 5-hydroxymethylcytosine (5hmC) was shown to be enzymatically produced in relatively high abundance in certain mammalian cells and tissues (Song et al. 2012). Following this discovery, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) were found in mouse embryonic stem cells (ESCs) and mouse tissues. These cytosine derivatives are produced from a stepwise oxidation of 5mC by the ten-eleven translocation (TET) family dioxygenases (Song et al. 2012).

5hmC accumulates with age and is most enriched in brain tissues (0.4–0.7 % of cytosine), where it is suspected to play regulatory roles in neurodevelopment and aging, as well as in neurological diseases (Kellinger et al. 2012). It exists in intermediate amounts (0.1 % of cytosine) in mouse ESCs where it may have dual roles of being a DNA demethylation intermediate as well as exhibiting gene regulation functions (Globisch et al. 2010). On the other hand, 5fC and 5caC are continuous

products of TET protein-mediated oxidation of 5hmC, and they can be removed by thymine DNA glycosylase (TDG). In contrast to 5hmC, 5fC and 5caC have not been found to accumulate in brain tissues. In fact, 5caC has been detected only in mouse ESCs, but not yet in other tissues (Kellinger et al. 2012). Currently, 5fC and 5caC are thought to be intermediates of active 5mC demethylation, although they may also have regulatory roles, which will be confirmed if specific binding proteins can be identified. For example, 5fC and 5caC were recently shown to slow down RNA polymerase II transcription, which may suggest possible functional interplay between transcription and these epigenetic modifications (Kellinger et al. 2012). To summarize, the discovery of these new nucleotide variants triggered an abundance of new information and new exciting ideas in the epigenetics field.

6.4.2 Posttranslational Histone Modifications (PTHM)

Chromatin is the state in which DNA is packaged within the cell. The nucleosome is the building block of chromatin and it is composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped (Kouzarides 2007). Histone proteins contain an N-terminal tail of 15–30 amino acids that protrude from the chromatin surface. This tail region is subject to modifications that include acetylation, methylation (mono-, bi-, or tri-), phosphorylation, ubiquitination, SUMOylation, deimination, and proline isomerization (Kouzarides 2007). These marks modify the local electrochemical properties of chromatin, altering its conformation and thereby regulating the accessibility of genes to the transcriptional machinery (Gapp et al. 2014). For the purposes of transcription, modifications can be divided into those that correlate with activation and those that correlate with repression. Acetylation, methylation, phosphorylation, and ubiquitination have been implicated in activation, whereas methylation, ubiquitination, SUMOylation, deimination, and proline isomerization have been implicated in repression (Kouzarides 2007). The balance between all modifications on various histone residues is kept by specific enzymes whose activities are tightly controlled since they ultimately determine the repertoire of proteins that are expressed in the cells (Hsieh and Gage 2005).

Perhaps the most studied PTHM are those that occur on the lysine residues on the free N-terminal tails. Of all the known modifications, acetylation has the most potential to unfold chromatin. This modification is typically associated with transcriptional activation, as it can physically relax the positive charge between the histone tail and the DNA backbone, enabling chromatin to open and providing access to the DNA for the transcriptional machinery (Kouzarides 2007). Additionally, the acetylation of lysine residues can lead to binding of bromodomain-containing proteins, which can recruit transcriptional activators (Dyson et al. 2001). Histone acetylation occurs via the activity of histone acetyltransferases (HATs), which include CREB-binding protein (CBP) and p300 (Day and Sweatt 2012). Acetyl groups are removed from lysine residues by histone deacetylases (HDACs), a large

family of proteins, which are organized into four different classes that differ in expression profiles across brain regions (Hsieh and Gage 2005).

Lysine residues can be mono-, di-, or even tri-methylated, with each distinct methyl group producing unique results (Scharf and Imhof 2011). Although H3K27 and H3K9 methylation is implicated in silent chromatin, the truth is likely to be that any given modification has the potential to activate or repress transcription under certain conditions. For example, methylation at H3K9 has a positive effect when it is found at the coding region and a negative effect when present at the promoter (Kouzarides 2007). These differential states are produced by tight regulation of the histone methylation machinery of which the histone methyltransferases (HMTs) and demethylases (HDMs) are the best characterized enzymes to date. (Day and Sweatt 2012). Many HDMs and HMTs are specific for modifications at individual amino acids on histone tails or even a specific number of methyl groups. For example, the HDM LSD1 (also known as KDM1) requires a protonated lysine to function and therefore cannot remove methyl groups from a trimethylated lysine (Stavropoulos et al. 2006). Moreover, HMTs such as G9a and SUV39H1 catalyze histone methylation, but not in a global manner as it appears to be the case for HATs (Greiner et al. 2005). G9a methylates lysine 9 on H3 (H3K9), which is generally a mark of repressed transcription (Tachibana et al. 2008). Likewise, MLL1 methylates H3K4, which is associated with transcriptional activation (Akbarian and Huang 2009).

6.5 Pomc Epigenetic Programming and Obesity: A Proof of Concept

Although behavioral adaptation is generally beneficial and helps adjust to a changing environment, it can also be maladaptive when external conditions and requirements change too rapidly and result in a mismatch with the adapted behaviors (Daskalakis et al. 2013). Such divergence between an individual's response and the surrounding milieu can lead to inappropriate and pathological behaviors and can increase the predisposition to disease (de Kloet et al. 2005). Energy homeostasis is reached in mammals by a mechanism that defines negative energy balance as its default state, unless a negative feedback signal is available upon elevated fat stores, indexed by increased leptin levels (Tups 2009). When this feedback mechanism is disrupted, the brain continuously "senses" a state of negative energy balance, promotes feeding, and reduces energy expenditure by default (Myers et al. 2010). Plasma leptin levels are higher in obese subjects, as expected from their higher fat mass compared with lean individuals (Schwartz et al. 2000). Thus, impaired response of the brain to the leptin signal has been shown to result in a persisting dysregulation of food intake and energy homeostasis (Tups 2009). The inability or reduced efficiency of leptin signaling to affect downstream physiological pathways, found in obesity, is termed "leptin resistance" (Myers et al. 2010). It was found that in common forms of obesity, humans and other animals become leptin resistant.

Several theories have been proposed for this phenomenon. One of them suggests that this disturbance might be mediated by epigenetic malprogramming.

A recent study that characterized the methylation pattern of the promoter of hypothalamic *Npy* in overfed pups (small litter model) did not observe any significant group differences (Plagemann et al. 2009). Rather, the analysis revealed global hypomethylation in all animals, i.e., independent of the mode of early nutrition. These data might therefore suggest a genuine resistance of the Npy promoter to environmentally induced modifications. This seems conceivable, considering that Npy is one of the most powerful orexigenic neuropeptides, which is vitally important for the survival of the individual (Gropp et al. 2005). Thus, it is possible that an epigenomic "fine-tuning" of food intake and body weight regulation is rather established via anorexigenic than orexigenic mechanisms. This would make sense, even from an evolutionary perspective, to ensure individual survival by adequate fuel supply as a primary and overall purpose (Plagemann et al. 2009).

In agreement with this theory, several studies have found new evidence pointing to the tight correlation between Pomc malprogramming and obesity. Results from our lab showed that both male and female postweaning rats, chronically exposed to a high-fat diet (HFD), developed an inability to compensate for their increased BW together with high levels of leptin and insulin. These elevated levels of hormones should on the one hand increase the expression of *Pomc*, leading eventually to increased energy expenditure and reduced BW, and on the other reduce expression of the orexigenic system (Cone et al. 1996; Cowley et al. 2001). Interestingly, the orexigenic system seems intact in those rats, since they demonstrated significantly lower *Npy* and *AgRp* mRNA levels, compared to lean, chow-fed controls. In contrast, both male and female obese rats presented deficits in the regulation of the anorexigenic system, while exhibiting no change (males) or decreased (females) levels of *Pomc* mRNA expression, indicating that high levels of leptin did not affect *Pomc* expression adequately. The prevailing view assumes that hypermethylation on CpG dinucleotides within gene promoters leads to blockage of transcription mechanisms and thus decreases mRNA expression (Haberman et al. 2012; Miranda and Jones 2007). In accord with this view, DNA-CpG methylation analyses revealed significantly higher levels of methylation in HFD compared to control fed rats at specific sites, including sites that affect Sp1 binding. Correlations between BW and leptin and DNA methylation levels in individual rats clearly indicated that hypermethylation of the promoter region is not "random" but appears on specific sites associated with leptin signaling. Moreover, ChIP assays showed that Sp1 failed to bind to the *Pomc* promoter in the hypermethylated HFD group (see Fig. 6.3). These results strengthen the argument that hypermethylation of the *Pomc* promoter region blocks the antiobesity actions of leptin by affecting transcription factor binding, such as Sp1 (Marco et al. 2013; Plagemann et al. 2009; Zhang et al. 2014).

In the same manner, using a combination of animal models, biochemical assays, and molecular biology, another study from Han's lab (Shi et al. 2013) showed that suppression of food intake with acute inflammation is not dependent on STAT3 activation in POMC neurons. Under these conditions, activated NF-κB independently binds to the *Pomc* promoter region between −138 and −88 bp (which also

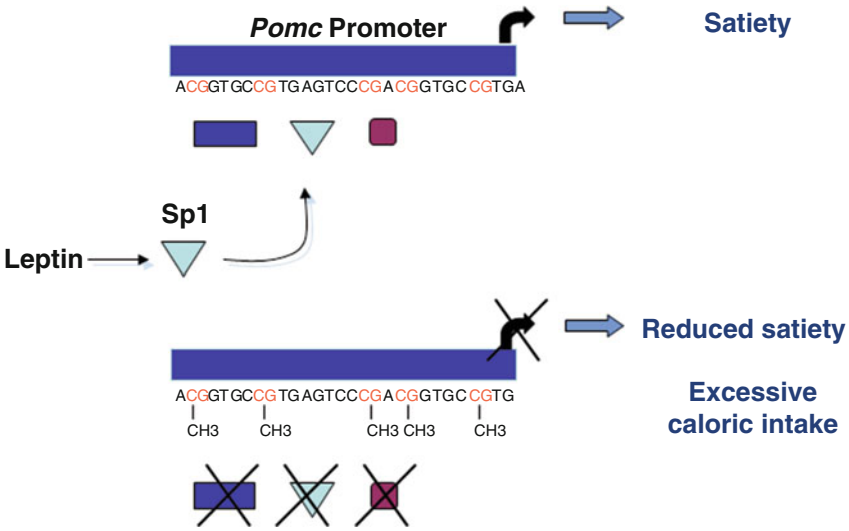

Fig. 6.3 Mediated by the hypothalamic leptin receptor (Ob-R) and insulin receptor (IR), high levels of leptin and insulin physiologically lead to an upregulation of hypothalamic expression of *Pomc*. This process occurs through a signal transduction mechanism that includes transcription factor binding, such as Sp1 to the *Pomc* promoter. Hypermethylation of the *Pomc* promoter region blocks the antiobesity actions of leptin by affecting Sp1 binding. Therefore, epigenetic modifications that lead to adjustment in this peptide's regulation might lead to reduced satiety and obesity

contains the SP1-binding site), leading to increased *Pomc* transcription. The authors show that chronic inflammation induced by HFD led to increased promoter methylation at the specific region on the *Pomc* promoter, which inhibited RELA binding, the most abundant subunit of NF-κB. Excess-free RELA blocks leptin-induced signaling by interaction with STAT3, leading to a reduced satiety signal and eventually to obesity (Shi et al. 2013). In addition, this group has provided two important insights. Firstly, by generating a series of deletion mutations in the promoter region of *Pomc*, the researchers found that mutations within the region between −138 and −88 bp were sufficient to abolish the RELA activity, pointing out the crucial role of this region in the transcription process. It is notable that HFD-treated mice presented hypermethylation at or near the RELA- and SP1-binding sites, compared with lean mice. Secondly, this paper shows high sequence homology of the *Pomc* promoter between different organisms, especially in the Sp1-/RELA-binding sites.

Another interesting paper recently reported on a mouse line with specific deletion of the MeCP2 gene in *Pomc* neurons. Researchers demonstrated that MeCP2 positively regulates *Pomc* expression in the hypothalamus. Absence of MeCP2 in Pomc neurons leads to increased DNA methylation of the *Pomc* promoter, which, in turn, downregulates *Pomc* expression, leading to obesity in mice with an accentuating degree of leptin resistance (Wang et al. 2014). There is also indirect evidence (not specific to the *Pomc* gene) showing changes in epigenetic mechanisms as a result of nutrition or diet manipulation. For example, a recent study has shown that histone deacetylases (HDAC) are involved in the epigenetic control of gene

expression and that they alter behavior in response to a variety of environmental factors. Specifically, this study examined the expression of HDAC family members in the medial hypothalamus of mice and found that in response to fasting, HDAC3 and −4 expression levels increased while HDAC10 and −11 levels decreased; 4 weeks on an HFD resulted in the increased expression of HDAC5 and −8. Moreover, fasting decreased the number of acetylated histone H3- and acetylated histone H4-positive cells in the ventrolateral subdivision of the ventromedial hypothalamus (Funato et al. 2011). Taking all these data together strengthens the argument that epigenetic modifications that lead to adjustment in this peptide's regulation can emerge at postweaning periods and might lead to or worsen the obesity phenotype.

Finally, another interesting finding regarding the plasticity of epigenetic mechanisms in adult subjects was found in post-lactating Wistar dams that were fed with HFD. Mated HFD dams, while heavier during pregnancy, demonstrated during 3 weeks of lactation a sharper BW decrease, lower leptin levels, and loss of hypermethylation on the *Pomc* promoter at weaning (Marco et al. 2014). Interestingly, weight loss during lactation was correlated with demethylation and with gadd45b mRNA expression levels, a protein recently suggested to have a potential role in active DNA demethylation (Sultan et al. 2012). Pregnancy and lactation are energy-consuming events, which naturally induce females to increase food intake and accumulate fat, yet the phenomenon of a sharp BW loss in obese dams during lactation has been described previously (Zagoory-Sharon et al. 2008). One reasonable explanation is that obese dams are challenged by the demands of rapidly growing pre-obese pups, using their energy stores for nursing. Thus, the mechanism of demethylation of the *Pomc* promoter, in response to extreme energetic challenges like lactation, cannot be ruled out. Several other enzymes, such as the TET family, have also been proposed as responsible for the demethylation mechanism (Rudenko et al. 2013), but much more research on this subject is needed.

6.6 Epigenetic Insights into Dysregulation of the Pomc System During Early Development

During early developmental phases, the brain experiences extensive growth remodeling (Finlay and Darlington 1995) and is particularly sensitive to external conditions and interference (Andersen 2003). It is assumed that the period of perinatal development is one of the most critical windows during which environment stimuli can affect the development of the fetus, as well as the offspring's postnatal health and behavior (Champagne and Meaney 2006; Symonds et al. 2009). It has been suggested that epigenetic mechanisms could represent the molecular mechanism underlying perinatal programming in those periods. Different studies showed that environmental alterations, such as maternal stress (Blaze and Roth 2013), undernutrition (Begum et al. 2013), smoking, and fetal alcohol exposure (Laufer et al. 2013; Novakovic et al. 2013) during sensitive periods affect gene expression in the offspring via altering epigenetic patterns. Of special relevance, the nutritional

environment during the perinatal period has been shown to alter the expression of genes critical to regulation of energy balance. It is important to note that the question of whether this programming is inherited through the gametes, during pregnancy or throughout lactation, is still not clear. However, there is some evidence that support both theories. For example, a recent paper showed that paternal obesity initiates metabolic disturbances in two generations of mice within a parental lineage and sex-specific manner, via alternations in the transcriptional profile of testis and sperm microRNA content. It was found that BW differences and metabolic deficit were maintained up to 17 weeks only in F2 male offspring (Fullston et al. 2013). On the other hand, studies have shown that manipulations carried out during late pregnancy (food-restricted dams) or lactation (small litters) were sufficient to change the hypothalamic set point via epigenetic mechanisms (Plagemann et al. 2009; Stevens et al. 2010; Waterland and Jirtle 2003). Notably such cases can easily mimic gametic transmission, since multiple generations, if timed appropriately, can be affected by a single exposure: the pregnant mother, the F1 embryo/fetus, and finally its developing germline and hence the future F2 generation (Youngson and Whitelaw 2008). In this chapter we review the three main models that were employed to test the maternal effect on epigenetic changes of the *Pomc* gene, in their offspring.

6.6.1 Overnutrition

One of the first sources of evidence that shows how epigenetic marks on the *Pomc* gene can undergo specific dynamic changes in response to early nutritional alternation came from Plagemann's group. This group used an animal model in which rats were raised in "small litters" (SL). Through artificial reduction of the natural litter size to only three pups per nest, SL neonates are subjected to early overfeeding, indicated, e.g., by early hyperglycemia, rapid fat accumulation, and obesity. Plagemann and colleagues showed that in SL rats the hypothalamic gene promoter of *Pomc* showed hypermethylation of CpG dinucleotides within two related transcription factor binding sequences (upstream to Sp1 and NF-κB) which are essential for the mediation of leptin and insulin effects on *Pomc* expression. The authors argue that Sp1-binding site itself does not contain any CpGs; however previous study shows that methylation of the region upstream of this sequence decisively influences Sp1-induced gene transcription (Zhu et al. 2003). Moreover, the inhibiting negative glucocorticoid receptor element (nGRE) binding sequence showed a nonsignificantly decreased methylation in SL rats.

Consequently, *Pomc* expression lacked upregulation, despite hyperleptinemia and hyperinsulinemia. Accordingly, the extent of DNA methylation within Sp1-related binding sequences was inversely correlated to the quotients of Pomc expression/leptin and Pomc expression/insulin, indicating functionality of acquired epigenomic alterations (Plagemann et al. 2009). A similar pattern of results was reported regarding the insulin receptor promoter (Plagemann et al.

2010). These results clearly point out the suckling period as one of the most critical "time windows" in which the nutritional environment can potentially lead to malprogramming and to an acquired new "set point" of energy balance in the offspring.

6.6.2 HFD and Dietary Supplementation

Insights into pathophysiological mechanisms responsible for long-term malprogramming due to exposure to maternal obesity before and/or during pregnancy and lactation are ideally addressed in experimental animals. A suitable animal model for the investigation of consequences of gestational overweight is the induction of maternal obesity by feeding a high-fat diet to rodents. Pregnant rat dams fed an HFD diet show increased weight gain during gestation and diet-induced obesity (Holemans et al. 2004). Very similar to the situation in offspring of obese mothers, the offspring of these rats develop hyperleptinemia, hyperinsulinemia, impaired glucose tolerance, as well as neonatal macrosomia (Holemans et al. 2004). This predisposition is intensified in animals genetically susceptible to obesity (Levin and Govek 1998; Walley et al. 2006). Nevertheless, and this is an important point to emphasize, there is a variable response of the offspring to diet-induced maternal obesity. Hence, outbred strains of animal models are highly popular to study obesity as they readily gain weight on high-fat diets. In particular, rats or mice have been studied for their ability to show a variable response to a high-fat diet (Farley et al. 2003). Some animals rapidly gain weight, while others gain only as much weight as those fed a low-fat diet. This phenomenon allows for the study of animals that are prone and resistant to obesity, the evaluation of individual differences, as well as finding the most suitable treatment to each individual (Farley et al. 2003; Holemans et al. 2004).

In a recent study, postnatal mice were reared by dams with dietary supplementation of conjugated linoleic acids (CLAs). The change of milk composition led to deficit in the regulation of the hypothalamic energy balance, expressed in increased of food intake, suppression of Pomc, attenuation of Sp1–promoter interaction, and the hypermethylation at the Sp1-binding site of *Pomc* promoter. In addition, a catch-up growth and adult metabolic changes like adult hyperglycemia and insulin resistance were observed in these postweaning pups (Zhang et al. 2014). Moreover, an in vitro model was used to scrutinize the Sp1–Pomc interaction in methylated or unmethylated CpGs of pGL3-POMC templates. Results show that the leptin-stimulated luciferase activity in the cells with a methylated promoter was significantly lower than that with an unmethylated promoter. In addition, EMSA analysis showed a DNA–protein complex only in the unmethylated promoter, indicating that Sp1 only bound to unmethylated DNA sequence. These observations provide insights into/on the importance of nutrition during lactation as a major factor that may induce a long-term metabolic dysregulation in the offspring.

In another study, we investigated the effect of perinatal exposure to maternal high-fat diet on weanling offspring, while focusing on DNA methylation alterations in the hypothalamic neuropeptide *Pomc* (Marco et al. 2014). Offspring of the HFD treated dams (HFD-C) presented high BW and leptin levels at weaning day. In addition, the HFD-C pups presented a significant reduction (~40 %) in both *Npy* and *AgRp* mRNA levels. In contrast, there were no group differences in *Pomc* mRNA expression; however the offspring to HFD dams presented low *Pomc*/leptin ratio compared to control offspring, indicating on impaired leptin/*Pomc* signaling. In accordance with their obesity phenotype, HFD pups presented hypermethylation across the entire *Pomc* promoter at PND 22. These results are consistent with the known physiological responses to unbalanced nutrition or excessive weight during pregnancy and lactation, since overeating (Plagemann et al. 2009), obesity, and even undernutrition (Begum et al. 2013; Stevens et al. 2010) were found to be high risk factors, both to the dam and her offspring. Thus, epigenetic malprogramming during the perinatal period may be one of the factors affecting the continuous increase in childhood obesity.

6.6.2.1 Reprogramming

It was previously suggested that epigenetic modifications that occur in critical time periods can have long-term effects on the offspring (Champagne and Meaney 2006; Laufer et al. 2013). Interestingly, although pups from both of the groups were weaned to standard rodent chow, HFD-C offspring in our study failed to maintain lean energy balance throughout their life, so that at adulthood they still presented increased intake and BW. More importantly, the hypermethylation pattern of HFD-C offspring was maintained at adulthood, across the entire *Pomc* promoter. Therefore it seems that maternal obesity leads to an acquired new "set point" of energy balance; hence obesity predisposition in these offspring was not "reprogrammed" by eating normal chow after weaning (Marco et al. 2014).

There are several studies in which epigenetic phenotype reprogramming has been reported following intervention during sensitive periods by changing the nutrition or the composition of the pup's diet (Burdge et al. 2009; Cho et al. 2013a). In the following section, these findings will be discussed in more detail.

In one study male offspring of dams fed high multivitamin (HV) diet during pregnancy weaned to recommended vitamin (HV-RV), high multivitamin (HV-HV), or tenfold folate (HV-HFol). Diets were compared with those born to RV dams and weaned to RV diet (RV-RV) for 29 weeks. Interestingly, only offspring diets similar to the mothers' diet lead to a more appropriate adaptation of the offspring to their postnatal environment compared to those fed an unmatched diet. HV-RV offspring presented obesity phenotype traits that include lower *Pomc* mRNA levels. Adding high levels of folate or multivitamins to the pup's diet prevented the increase in food intake and body weight and normalized *Pomc* expression to the RV-RV levels (Cho et al. 2013b). Neither the gestational nor postweaning diet had any effect on the global methylation status in the offspring at 29 weeks

postweaning. However, in the following study, the researchers used the same protocol; only this time the dam's diet included HFol (instead of HV). In the same manner, the HFol-RV offspring were heavier, with low *Pomc* mRNA levels. HFol supplement after weaning corrected these parameters to normal values; only this time it was associated with decreased methylation levels on the hypothalamic *Pomc* promoter (Cho et al. 2013a). Folate is a methyl metabolism cofactor in processes of DNA methylation, which in turn can modify gene expression and function in regulatory systems. Therefore, these results indicate that epigenetic plasticity of the hypothalamus responds to folate consumption not only in utero but also in later life.

Another interesting study showed that leptin supplementation of neonatal rats during the suckling period protects against being overweight in adulthood and ameliorates the control of food intake. This was associated with changes in the expression of *Pomc*, leptin receptor (*Lepr*), and suppressor of cytokine signaling (*Socs3*). In addition, *Pomc* promoter methylation increased in control animals fed with HFD but decreased in leptin-treated animals, indicating that leptin treatment during the postnatal, suckling period may program methylation on the *Pomc* gene, with possible implications for gene expression and protection against the development of obesity (Palou et al. 2011).

In our study, 2 months of postweaning normal nutrition did not alter the obese phenotype of the rats raised by HFD-fed dams as these mature offspring retained significantly high BW and leptin levels as well as hypermethylation at the *Pomc* promoter. In the studies mentioned above, the dams were fed during pregnancy/lactation with either high multivitamin or high folate. Therefore, a reasonable explanation is that these diet manipulations are not as "severe" as the 60 % fat diet used in our study and thus can be more readily reprogrammed. Moreover, in the Palou et al. study, leptin treatment was administered during the suckling period, whereas in our study the attempt to reprogram the F1 offspring occurred during the postweaning period. These differences between studies indicate that the hypothalamic system is more accessible to epigenetic programming during the suckling period. This may be why our attempt to "repair" after this period was ineffective. Future treatment will have to take into account the intensity and the optimal time window for intervention. Another interesting point is that the offspring to HFD-treated dams revealed an age-related increase of DNA methylation from ~50 % at PND 21 to nearly 80 % at PND 90 (in the most hypermethylated sites). Growing evidence suggests that epigenetic mechanisms, such as DNA methylation, affect gene expression also in an age-dependent manner (Fraga and Esteller 2007; Wu et al. 2014). Therefore, there may be a natural process in which mechanisms, such as maintaining energy balance, lose their effectiveness toward old age. Taking all this together suggests that the impact and long-term consequences of the nutrition are dependent on the "time window," the duration, and the intensity of the exposure (Table 6.1).

Table 6.1 Epigenetic phenotype reprogramming of obesity

Model organism	Environmental exposure	Environmental manipulation	Epigenetic reprogramming	Epigenetic changes	Reference
Wistar rats (males)	Dams fed with either high multivitamin (HV) diet or recommended vitamin (RV) diet, during pregnancy	Offspring weaned to recommended vitamin (HV-RV), high multivitamin (HV-HV), or tenfold folate (HV-HFol) diets and were compared with those with RV-RV for 29 weeks	No	–	Cho et al. 2013b
Wistar rats (males)	Dams fed with tenfold folate (HFol) diet during pregnancy	Offspring were weaned to recommended vitamin (HFol-RV) or HFol (HFol-HFol)	Yes	HFol supplement after weaning corrected obesity parameters to normal values that was associated with decreased methylation levels on the hypothalamic Pomc promoter	Cho et al. 2013a
Wistar rats (males)	Male rats were treated daily with an oral physiological dose of leptin or vehicle during the suckling period	After weaning, animals were fed with a normal-fat or a high-fat (HF) diet until aged 6 months	Yes	Pomc promoter methylation increased in control animals fed with HFD but decreased in leptin-treated animals	Palou et al. 2011
Wistar rats (females)	Perinatal exposure to maternal high-fat diet (60 %)	Pups from both of the groups were weaned to standard rodent chow	No	–	Marco et al. 2014

6.6.2.2 Intrauterine Growth Retardation IUGR (Undernutrition)

Both pre- and postnatal under nutrition, similar to the effects of over nutrition, produce long-term consequences on food consumption leading to obesity later in life (Levin 2006). The apparent paradox whereby both excess and deficiency of energy supply during gestation are linked to offspring obesity is mirrored in the fact that insulin deficiency (type 1 diabetes mellitus, T1DM) and insulin excess (T2DM) during pregnancy both increase the risk that offspring will develop obesity and T2DM (Silverman et al. 1991). Hence, disruption of the delicate energy balance during development leads to metabolic imprinting, inducing a resetting of the set point that defines satiety (Waterland and Garza 1999).

Epidemiological studies in humans suggest that undernutrition during various phases of gestation can predispose some individuals to become obese and insulin resistant. The most influential studies have been on the Dutch famine. The tragic circumstances of the Dutch Hunger Winter of 1944–1945 created a unique opportunity to study the relation between exposure to prenatal famine and health in adult life (Kyle and Pichard 2006). Among many phenotypes ranging from heart disease to schizophrenia, which were correlated to this 5-month period of severe malnutrition, there was a long-term metabolic phenotype. People born after exposure to famine in late and mid-gestation were lighter, shorter, and somewhat thinner at birth. There was low glucose tolerance in these people, which was more severe than could be explained by the famine-related reduction in birth size. Maternal malnutrition during early gestation was associated with higher body mass index and waist circumference in 50-year-old women but not in men. Furthermore, a clear correlation was established between the size of a fetus (which in many cases results from maternal malnutrition) and metabolic syndromes later in life. Offspring who weighed less than 2.5 kg at birth are 7 times more likely to be glucose intolerant or type 2 diabetic than those who weighed more than 4. 3 kg at birth (Hales et al. 1991).

Animal models were developed to study the effect of maternal undernutrition on the metabolism of their offspring, including rodents, piglets, sheep, and baboons. All demonstrate increased body weight throughout life (Sarr et al. 2012). Pregnant rat dams that were fed a low-protein diet during pregnancy and lactation produced pups suffering from a reduced birth weight of approximately 15 %, and the reduction in body weight of the offspring was maintained throughout life. Nevertheless, in early adult life, the low-protein offspring were more glucose tolerant and insulin sensitive. There was, however, an age-dependent impairment in glucose tolerance; by 15 months of age, there was markedly worsened glucose tolerance and by 17 months the male low-protein offspring were diabetic with elevated insulin levels. The deterioration can be accelerated by high-fat feeding (Stocker et al. 2005).

Adult male IUGR offspring from prenatally 70 % food-restricted rat dams throughout gestation were predisposed to energy balance dysfunctions such as impaired glucose intolerance, hyperleptinemia, hyperphagia, and adiposity (Lukaszewski et al. 2013). Low-birth-weight piglets also display significantly higher body fat at 12 months compared to normal-birth-weight piglets (Poore and

Fowden 2004). In a nonhuman primate, i.e., baboons, maternal nutrient reduction during pregnancy and lactation programs offspring metabolic responses, increasing insulin resistance and β-cell responsiveness, resulting in emergence of an overall phenotype that would predispose to later life type 2 diabetes (Choi et al. 2011).

The development of the hypothalamic satiety circuit differs between human and rodents. While in the former most of the development is prenatal, in rodents the development occurs during the first postnatal 2 weeks. The development of energy homeostasis is mediated by central neural systems, which are genetically determined. Nevertheless, in rodents during a postnatal critical period, leptin plays a neurotrophic role whereby a leptin surge shapes the neuronal pathway connecting the ARC to the PVN (Bouret and Simerly 2006). However, these systems are highly plastic and readily modified by environmental inputs. In particular, it was shown that maternal undernutrition prevents the postnatal surge of plasma leptin, disturbing preferentially the hypothalamic wiring of the anorexigenic POMC cells, in male rat pups (Delahaye et al. 2008).

In addition, to the hypothalamic wiring adjustment caused by IUGR in the offspring, maternal undernutrition affects also the molecular pathway underlying metabolic homeostasis. The expression levels of both anorexigenic and orexigenic ARC neuropeptides are altered. Depending on the intensity of the insult, 50 % maternal feed restriction in rats caused a significant reduction in *Pomc* expression in the offspring from PND14 until PND30, while *Npy* gene expression was not significantly modified (Delahaye et al. 2008). In sheep, moderate levels of maternal undernutrition during early gestation did not affect the expression levels of neither *Pomc* nor *Npy* (Begum et al. 2013; Stevens et al. 2010). Nevertheless, maternal undernutrition effects in primates are clear. It was reported that intrauterine growth restriction altered fetal baboon hypothalamic appetitive peptide balance, causing an increase in the expression of *Npy*, GR, and active phosphorylated GR and decreased expression of *Pomc* and the phosphorylated form of STAT3 (Li et al. 2013).

Since the long-term maternal effect on the metabolic set point of their offspring depends on alteration in the expression of the ARC neuropeptides, it was pertinent to hypothesize the involvement of epigenetic regulation. Indeed, initial screening of CpG methylation at the *Pomc* promoter of offspring of feed-restricted rats revealed less methylation at specific sites in the promoter area between the TATA box and the transcription start site (Coupe et al. 2010). Corroborating evidence for epigenetic regulation of POMC in offspring as a result of maternal undernutrition can be found in a study demonstrating H3K9 hyperacetylation and hypomethylation in sheep (Stevens et al. 2010). It should be noted that in this model IUGR treatment did not have an effect on the expression of *Pomc* in the offspring. Additional evidence of the involvement of epigenetic regulation in the satiety balance set point can be found in a study using the sheep model which demonstrates that in prenatal maternal undernutrition exposed sheep, there was decreased glucocorticoid receptor (GR) promoter methylation, decreased histone lysine 27 trimethylation, and increased histone H3 lysine 9 acetylation in hypothalami of male and female adult offspring (Begum et al. 2013).

6.7 Pomc Epigenetic Marks as a Biomarker in Humans: Clinical Implications

Epigenetic marks on the *Pomc* promoter may be especially relevant to humans, since recently published data show that epigenetic changes in the brain, specifically CpG methylation of the gene promoter, may be reflected in other cells, such as white blood cells. Therefore, these marks may be used as biomarkers for early detection and prevention of obesity. For example, an identical pattern of *Pomc* methylation was found in postmortem dissected POMC neurons and peripheral blood cells from the same patient (Kuehnen et al. 2012). Another study presented an association of weight regain with specific methylation levels in the *Npy* and *Pomc* promoters in leukocytes of obese men (Crujeiras et al. 2013). Therefore, methylation pattern of the *Pomc* promoter may potentially be a prognostic biomarker for early detection and prevention of obesity (Table 6.2).

6.8 Summary

The question remains *how do* the nutritional content and composition of the diet induce epigenetic alterations? Two potential mechanisms should be proposed here. First, hyperleptinemia, as occurs due to overfeeding or HFD diet, induces peripheral as well as central nervous system leptin resistance (Bjorbaek et al. 1999), which leads to increased levels of transcription factors such as Sp1, STAT3, and ERα (Bates et al. 2003; Gao and Horvath 2008; Zhang et al. 2014). These factors are known for their ability to recruit enzymes such as DNMT (Hervouet et al. 2009) and histone methyltransferase (Zhang et al. 2013) which, in turn, establish the epigenetic marks. Moreover, hyperleptinemia induced the action of proteins such as FoxO1 and Socs3 to block the Sp1/STAT3 complex (Yang et al. 2009) and therefore increased accessibility of the Sp1-/STAT3-binding site to enzymes such as methyltransferases, possibly leading to increase methylation. Second, hyperglycemia has recently been shown to increased DNA methyltransferase activity and subsequent DNA methylation, leading to glucose-dependent hypermethylation (Chiang et al. 2009). Since central leptin resistance and hyperglycemia typically accompany DIO as crucial features, a combination of these two alterations seems to be a particularly plausible and potentially even additive mechanism of acquired epigenetic alterations.

Table 6.2 Environmental exposure effect epigenetic programming of the hypothalamic Pomc gene

Model organism	Age	Environmental exposure	Behavioral alterations	Epigenetic mechanism involved	Epigenetic changes	Reference
Wistar rats	PND 90	60 % high-fat diet (HFD), 10 weeks	Obesity, hyperleptinemia, hyperinsulinemia	DNA-me	Hypermethylation on the Pomc promoter in HFD males group	Marco et al. 2013
C57Bl/6 mice	Adulthood	60 % high-fat diet (HFD), 20 weeks		DNA-me	HFD-treated mice presented hypermethylation at the specific region on the Pomc promoter, which inhibited RELA binding. Excess-free RELA blocks leptin-induced signaling	Shi et al. 2013
Mice	Adulthood	MeCP2 deletion in hypothalamic Pomc neurons	Overweight, increased fat mass, increased food intake, respiratory exchange ratio, hyperleptinemia	DNA-me	Deletion of MeCP2 in POMC neurons leads to increased DNA methylation of the hypothalamic Pomc promoter and reduced Pomc expression	Wang et al. 2014
Mice	Weaning	Postnatal mice were reared by dams with dietary supplementation of conjugated linoleic acids (CLAs)	Changes of milk composition in dams Deficit in the regulation of the hypothalamic energy balance, increased of food intake A catch-up growth and adult metabolic changes like adult hyperglycemia and insulin resistance were observed	DNA-me	Suppression of Pomc, attenuation of Sp1–promoter interaction, and hypermethylation at the Sp1-binding site of Pomc promoter	Zhang et al. 2014

(continued)

Table 6.2 (continued)

Model organism	Age	Environmental exposure	Behavioral alterations	Epigenetic mechanism involved	Epigenetic changes	Reference
Wistar rats (females)	Weaning	Perinatal exposure to maternal high-fat diet (60 %)	Obesity, hyperleptinemia	DNA-me	HFD pups presented hypermethylation across the entire Pomc promoter	Marco et al. 2014
Wistar rats	PND 21	Overfed pups throughout lactation—small litter model	Obesity, hyperleptinemia, hyperglycemia, hyperinsulinemia, and an increased insulin/glucose ratio	DNA-me	Pomc—hypermethylation in SL pups NPY—no significant group differences, hypomethylation in all animals	Plagemann et al. 2009
Ewes	Adulthood	Maternal under nutrition (10–15 % body weight reduction)		PTHM	In fetuses from ewes undernourished, there was increased histone H3K9 acetylation and hypomethylation of the POMC gene promoter but no change in POMC expression	Stevens et al. 2010

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Chapter 7
Alterations in DNA Methylation and Hydroxymethylation Due to Parental Care in Rhesus Macaques

Nadine Provencal, Renaud Massart, Zsofia Nemoda, and Stephen Suomi

Abstract Early life is one of the most important and sensitive periods during the development of an individual. During this stage, the body and especially the brain are known to be greatly responsive to environmental cues, such as the early social environment. As a consequence, early life adverse social experiences in humans are associated with a wide range of health problems in adulthood. The broad range of phenotypes associated with early life stress (ELS) suggests a system-wide response of the organism, which is yet to be determined. In the last decade, increasing evidence suggests that epigenetic mechanisms underlie the effects of ELS on adult human health. However, there are critical challenges in delineating the direct effects of ELS on epigenetic profiles and phenotypes in human studies. It is impossible to randomize ELS and rare are the studies where complete information about past environmental insults is available, which would allow us to conclude on causality. Nonhuman primates offer several advantages in addressing these challenges. This chapter focuses on parental deprivation models in rhesus macaques which have been shown to produce an array of behavioral, physiological, and neurobiological

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deficits that parallel those identified in humans subjected to ELS. It describes the evidence for epigenetic alterations induced by differential rearing in this model and points out the differences between tissue-specific versus multi-tissue changes and outlines possible mechanisms for these to occur. In addition, it highlights the need for multi-omics longitudinal studies to better understand the epigenetic trajectories induced by ELS exposure and their impact on adult health.

Keywords DNA methylation • DNA hydroxymethylation • Parental care • Rhesus macaques • G x E • Genetic and Epigenetic

7.1 Introduction

Early life is one of the most important and sensitive periods during the development of an individual (Lupien et al. 2009). During this stage, the body and especially the brain are known to be greatly responsive to environmental cues since they undergo dynamic changes (Bock et al. 2014). As a consequence, early life adverse social experiences in humans are associated with a wide range of health problems such as increased reactivity to stress, cognitive deficits, psychiatric and behavioral disorders (O'Connor et al. 2005; Loman et al. 2010; Heim et al. 2012), as well as increased vulnerability to develop chronic diseases in adulthood such as metabolic and cardiovascular diseases (Power et al. 2007). The broad range of phenotypes associated with early life stress (ELS) suggests a protracted system-wide response of the organism, which still needs to be determined. In the last decade, increasing evidence suggests that epigenetic mechanisms, in line with their role in cellular programming, underlie the effects of ELS on adult health. Indeed, a growing number of studies have reported that the epigenome is responsive to external environmental exposures including the social environment both in humans (Mill et al. 2008; Szyf 2012; Sasaki et al. 2013; Klengel et al. 2014; Nieratschker et al. 2014; Provencal et al. 2014a) and in animal models (Darnaudery and Maccari 2008; Gudsnuk and Champagne 2012). For example, in rats, low maternal care induced long-lasting changes in the pups' epigenetic profile of the glucocorticoid receptor gene in the hippocampus, which conferred higher stress reactivity in adulthood (Weaver et al. 2004). Maternal separation in mice was also shown to induce long-lasting epigenetic alterations in the vasopressin gene of the paraventricular nucleus, which conferred altered stress response in these pups (Murgatroyd et al. 2009). It has therefore been hypothesized that the epigenome, shaped by the early environment, can influence stress reactivity of an individual and the risk or resilience to develop diseases later in life (Szyf and Bick 2013; Zannas et al. 2015).

There are critical challenges in expanding the initial findings in animals, where causality could be tested, to humans. It is impossible to randomize ELS in humans, and therefore it is hard to tell whether the differences observed are driven by innate genetic differences or whether they are mainly environmentally driven or come from an interaction between both. Moreover, rare are the studies where we have complete

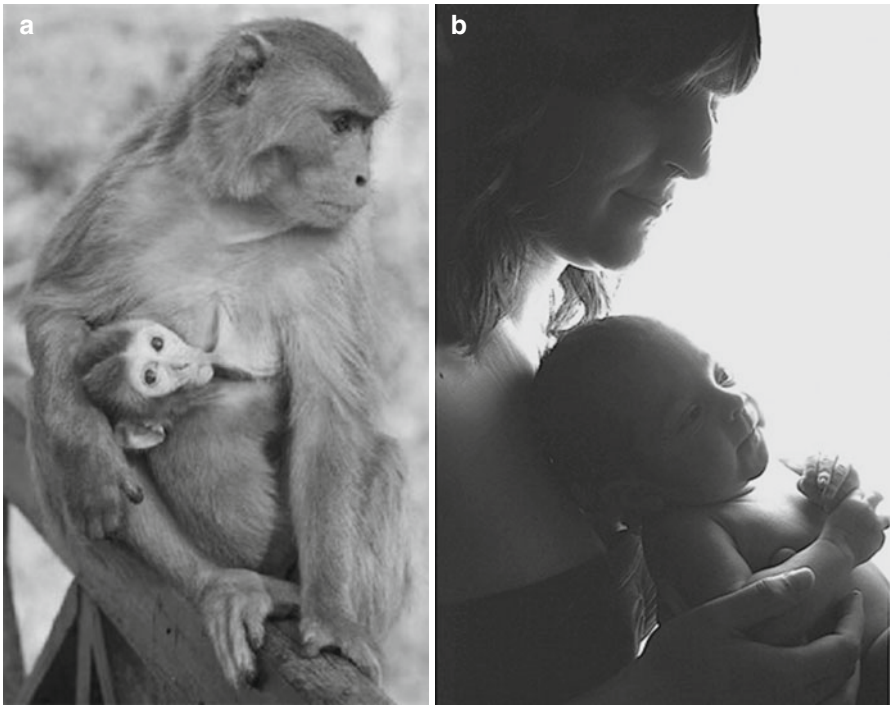


Fig. 7.1 Picture of (**a**) *Macaca mulatta* and (**b**) *Homo sapiens* mothers nursing their infants in their natural environments

information about any past environmental insults, such as infections and metabolic or psychological stressful events, allowing us to conclude on causality. Nonhuman primates, in addition to their close evolutionary relationship and similar phenotype to humans (Fig. 7.1), offer several advantages in addressing these challenges. First, they provide an exceptional opportunity to dissociate the impact of early life stressors on the epigenome from genetic causes, by randomization of the groups prior to experimental application of ELS, which is impractical in humans. Second, under laboratory housing, it is possible to separate "background" environmental factors, such as access to food, from the social component per se. Third, certain species of monkeys can have genetic variants functionally similar to that of humans, e.g., at the serotonin transporter gene, offering a possibility for controlled gene x environment (G×E) interaction analyses in monkey colonies, which are easily translatable to humans.

Optimal social environment, particularly sensitive maternal care, and the resulting secure mother–infant bond are critical for infant development in primates and are required for developing appropriate social skills later in life (Suomi 1997). In rhesus monkeys (*Macaca mulatta*), the mother–infant bond during the first months of life is very similar to the human mother–infant attachment (Suomi et al. 2008). Therefore, studying the behavioral and biological effects of suboptimal maternal care or maternal separation on infant development in rhesus monkeys can potentially bring translatable results about ELS to humans. Several models of adverse social environment

have been developed and studied in rhesus macaques in relation to psychiatric disorders, although naturally occurring abusive parenting can also be studied in this species (Parker and Maestripieri 2011). In this chapter, we will focus on the models of parental deprivation (nursery and peer rearing) that have been repeatedly shown to produce an array of behavioral, physiological, and neurobiological deficits that parallel those identified in humans subjected to early life adversity (Harlow and Harlow 1965; Sackett 1965, 1984; Kaufman and Rosenblum 1969; Kraemer 1992; Suomi 1997, 2011; Machado and Bachevalier 2003; Bennett and Pierre 2010). Next we will describe some of the evidence for epigenetic alterations induced by differential rearing in rhesus macaques. We will also highlight the importance of tissue specificity versus global changes and timing of the stressful exposure.

7.2 Long-Lasting Effects of Early Social Environment in Rhesus Macaques

In the maternal deprivation model, rhesus monkeys are randomly assigned to different rearing conditions at birth. The control infants stay with their biological mothers in a large social group (MR = mother reared), whereas socially deprived monkeys are raised individually in a nursery for a month (NR = nursery reared). After the first month, these NR infants are either placed together with their peers in small groups (PR = peer reared) or left alone in their cage with an inanimate, cloth-covered surrogate mother for most of the day, allowing 2 h per day of interaction with their peers (SPR = surrogate–peer reared) until 6–12 months of age (for detailed description of this model, see Conti et al. 2012). The PR and SPR conditions serve as a controlled and relevant early life stressor (before the end of their first year, all monkeys are placed in a mixed social group). The treatment period in this rhesus macaque model is equivalent to the first 3 years of human life, which is a critical period for appropriate development.

The maternal deprivation model (which allows some form of social contact) is reliably used model to study depressive and anxiety disorders based on the similarities of insecure mother–infant attachment, leading to emotional and social disturbances and behavioral abnormalities also observed in humans (Dettmer et al. 2014). Compared to MR monkeys, both PR and SPR monkeys show higher prevalence and frequency of motor stereotypes, but there is an interesting sex difference: male SPR monkeys have even higher rate of stereotypic behaviors compared to PR males, whereas there is no further increase in behavioral abnormalities among female SPR and PR macaques (Conti et al. 2012). There are other important differences between the two sexes in this model, usually male SPR rhesus monkeys are much more affected by social deprivation, leading to increased frequency of illnesses later in life and higher mortality rate (Conti et al. 2012; Lewis et al. 2000), possibly because of impaired cellular immunity (Coe et al. 1989; Lewis et al. 2000). Although PR monkeys develop strong bonds with their peers, they show higher levels of anxiety when compared to MR monkeys. Since PR macaques are raised in the absence of

adult care, without potential punishment of inappropriate behaviors, they often have impaired behavioral inhibition system, leading to highly reactive and aggressive behavior (Suomi 1997; Dettmer and Suomi 2014). Based on these observed behavioral abnormalities, other frequently studied psychopathologies seen in the maternal deprivation model are related to externalizing disorders, like conduct disorder or antisocial personality disorder, and to substance use disorders, such as alcoholism.

7.2.1 Physiological and Behavioral Changes

Disruptions of social relations, especially the lack of maternal care in the first month of the rhesus macaque life (NR condition) and the absence of a secure mother–infant attachment which would allow the infant to safely explore the environment (PR and SPR condition), result in a wide range of internalizing and externalizing behavior problems (reviewed by Stevens et al. 2009). PR macaque infants show increased anxiety; they engage less in social play and explore less. As adults, PR monkeys continue to have less social interactions; as a consequence they have a lower social rank than their MR counterparts, where SPR macaques usually rank at the bottom of the social dominance hierarchy (Bastian et al. 2003). The resulting neuroendocrine changes, e.g., altered stress reactivity in these animals, can potentially serve as the mediating factor for the majority of the long-lasting effects of ELS, such as impaired cell-mediated immune activity, higher rate of illnesses, and decreased survival rate (Conti et al. 2012; Lewis et al. 2000). Even years after the social deprivation period, PR monkeys exhibit delayed cortisol activation to acute stressor and behavioral abnormalities, such as increased stereotype behavior and decreased locomotion and social affiliative behavior (Feng et al. 2011). The effects of stress have also been demonstrated in specific brain regions, such as the dorsomedial prefrontal and anterior cingulate cortex. PR juvenile monkeys show enlargement in these stress-sensitive brain regions (Spinelli et al. 2009). Changes in the stress regulatory system can also affect ethanol sensitivity and alcohol consumption through the actions of neuroactive steroids (Morrow et al. 2006), rendering PR monkeys more prone to alcohol abuse (Dettmer and Suomi 2014).

In addition to corticosteroid changes, stressful events can affect monoamine neurotransmitter systems, which are important modulators of emotional responses and arousal. In relation to norepinephrine (NE), PR monkeys have increased level of its metabolite (3-methoxy-4-hydroxyphenylglycol: MHPG) in the cerebrospinal fluid (CSF) and an attenuated NE response after stressful events (separation and group formation) compared to MR infants (Clarke et al. 1996). In contrast, lower levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) were reported in PR and SPR infants in a large sample of rhesus monkeys (Shannon et al. 2005). These alterations in neurotransmitter metabolites mirror the abnormalities in NE and serotonin functioning observed in patients with impulsive aggression (Oquendo and Mann 2000) and explain in part the increased aggressive behaviors seen among PR monkeys. It is interesting to note that higher levels of aggressive and impulsive

behaviors were also observed in free-ranging macaques with low 5-HIAA levels in CSF (Howell et al. 2007; Westergaard et al. 2003). Furthermore, neurotrophins, such as brain-derived neurotrophic factor (BDNF), are also affected by early social deprivation in monkeys. Increased plasma BDNF levels were observed in PR (but not SPR) infants compared to controls. In addition, a genetic polymorphism in the pro-BDNF domain was shown to affect the BDNF levels, suggesting that both genetic and environmental factors can regulate the blood level of this neurotrophic factor (Cirulli et al. 2010). It seems that plasma BDNF could serve as a peripheral marker of brain plasticity, although plasma neurotrophins can originate from lymphocytes and endothelial cells in addition to the diffusion from the brain (Cirulli et al. 2009). In conclusion, it is likely that maternal deprivation induces multiple neurological alterations that interact to shape the developing brain (see reviews by Homberg et al. 2014 and Lanfumey et al. 2008), accounting for the long-lasting effects in PR and SPR macaques' behavior.

7.2.2 Altered Stress Reactivity

The most important system in stress regulation is the hypothalamic–pituitary–adrenal (HPA) axis. Upon activation, corticotropin-releasing hormone (CRH) and vasopressin (AVP) are released from the hypothalamus and stimulate adrenocorticotropic hormone (ACTH) release from the pituitary into the bloodstream. ACTH in turn activates the secretion of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex into the blood. Cortisol has effects on many tissues including the brain where it exerts a negative feedback loop on CRH/AVP and ACTH release stopping the activation of the HPA axis. The cellular actions of cortisol are mediated by its binding to the glucocorticoid receptor (GR) and the mineralocorticoid receptor (Larsson et al. 2012), which act as transcription factors regulating adaptive responses to stress, including metabolism, immune activation, and cell proliferation and differentiation. For example, activated GR can act as a repressor of pro-inflammatory cytokine genes through its binding to glucocorticoid response elements (GREs) in regulatory regions of these genes. In addition, corticosteroids can also signal through membrane-associated receptors and exert rapid, non-genomic effects in the brain altering the excitability of neurons (Groeneweg et al. 2008).

It has been shown in rodent models that lower levels of maternal care induce a decrease in GR expression through epigenetic changes in the offspring hippocampus and lead to an impaired negative feedback (Weaver et al. 2004). Human studies also showed long-lasting effects of childhood abuse at the *GR* gene (*NR3C1*): childhood abuse decreased GR expression through increased DNA methylation at the *NR3C1 1 F* gene promoter in adult hippocampus (McGowan et al. 2009). Such early life adversities were also shown to associate with DNA methylation alterations at the *NR3C1* promoter in leukocytes of adults and adolescents and even in cord blood samples (see reviews by Szyf and Bick 2013, and the respective chapters of this

book series). In contrast, GREs located within the *FK506 binding protein 5* (*FKBP5)* gene were shown to be demethylated after chronic application of corticosterone in mice (Lee et al. 2010), as well as abuse in childhood and after dexamethasone treatment in human hippocampal progenitor cells (Klengel et al. 2013). Excessive release of glucocorticoids following ELS could specifically alter the neuronal structure of glucocorticoid-sensitive areas of the brain and induce epigenetic alterations either through direct binding of GR to GREs or through activation of neuronal signaling pathways regulating epigenetic proteins such as MeCP2 (Murgatroyd et al. 2009). Indeed, activated GR has been shown to induce stable DNA demethylation in and around GREs, leading to increased transcriptional sensitivity of the target gene (Thomassin et al. 2001; Lee et al. 2010; Grontved et al. 2013). For more information on glucocorticoid-dependent epigenetic effects, please refer to Chap. 2 of this book.

In rhesus macaque models, it was shown that early life adversities in the form of maternal deprivation or abusive parenting could cause alterations in the HPA axis. For example, lower plasma ACTH and cortisol levels were observed at baseline in PR infants compared to MR (Barr et al. 2004b). It is important to mention that gender and serotonergic genetic variations were also shown to have an effect on the stress system in addition to rearing conditions (Barr et al. 2004a). These G × E interactions are described in detail in the next section of this chapter. In addition, since cortisol exhibits a diurnal rhythm, only measuring basal cortisol levels at one time point might not give a replicable result. Therefore, measuring stress reactivity by comparing cortisol (and/or ACTH) levels over a period of time might be more sensitive in detecting HPA axis dysfunction. Under experimental settings, either a stressful situation (e.g., separation from their social group or introduction to a new group) or pharmacological tests (dexamethasone suppression test and the ACTH or CRH stimulation tests) can be used to better measure HPA axis regulation. Another way to circumvent the problem of unstable blood cortisol levels is to measure cortisol in hair where it accumulates over weeks/months (Feng et al. 2011). Using these more appropriate measures, blunted cortisol response was observed in NR infant macaques following separation or pharmacological challenge (Barr et al. 2004b; Capitanio et al. 2005). The picture was more complex with the comparison of hair cortisol samples of MR vs. PR or SPR infants before and after their differential rearing treatment period, probably due to the natural decline of hair cortisol level over the years. Indeed, 10 months after their relocation, at 18 months of age, higher hair cortisol levels were measured in both PR and SPR monkeys compared to MR, but by 24 months of age, any difference disappeared (Dettmer et al. 2012). However, decreased cortisol levels were measured from the hair of PR monkeys at 2 and 3.5 years of age in a previous study (Feng et al. 2011). Although the noninvasive sampling method for cortisol assessment is quite interesting, these findings should be taken cautiously because of technical issues affecting hair as a biological sample, such as water exposure (see Hamel et al. 2011) and small sample sizes in these studies. Taken together, these HPA axis alterations among socially deprived macaques, even if they are present only during the first few postnatal months, have important neurobiological consequences through direct effects of GR-mediated actions and

Table 7.1 Neurobiological changes in maternally deprived macaques. Overview of the biological changes in the rhesus monkey (*Macaca mulatta*) maternal deprivation model, differences between *MR* mother reared, and *NR* nursery reared, *PR* peer reared, *SPR* surrogate–peer-reared conditions

Altered biological system	Effected molecule	Differences between PR or SPR (NR) and MR rhesus macaques	Reference
Changes in CSF monoamine metabolite levels	Norepinephrine (MHPG)	PR infants had increased MHPG level and attenuated NE response to stress	Clarke et al. 1996
	Serotonin (5-HIAA)	PR and SPR infants had lower 5-HIAA from age 2 weeks to 5 months	Shannon et al. 2005
Changes in neurotrophin levels	BDNF NGF	PR female infants had increased plasma BDNF at 2 months PR male infants had increased plasma NGF at 2 months of age	Cirulli et al. 2009
Altered stress hormone levels	Cortisol	Plasma cortisol was lower in NR infants at baseline and after stress at 3–4 and 6 months Hair cortisol was higher in PR and SPR monkeys at 18 months (not at 2 years) Hair cortisol was lower in PR monkeys at 2 and 3.5 years of age	Barr et al. 2004b Capitanio et al. 2005 Dettmer et al. 2012 Feng et al. 2011

5-HIAA 5-hydroxyindoleacetic acid, *BDNF* brain-derived neurotrophic factor, *CSF* cerebrospinal fluid, *MHPG 3*-methoxy-4-hydroxyphenylglycol, *NE* norepinephrine, *NGF* nerve growth factor

indirect effects by moderating crucial neurotransmitter systems, such as the serotonergic system (see Table 7.1 for an overview).

7.2.3 Gene × Environment Interaction at the Serotonin Transporter

The neurotransmitter serotonin (5-HT: 5-hydroxytryptamine) is involved in the regulation of various processes such as food intake, sleep, and mood as well as brain development. In addition, 5-HT can modulate the response to stress by interacting with the HPA axis. Based on findings in rhesus macaques related to the brain-specific tryptophan hydroxylase (TPH2, catalyzing the rate-limiting step in 5-HT synthesis), Chen and Miller (2012) described in detail a model implying reciprocal interactions between stress regulation and serotonergic system. Specifically, they proposed that release of cortisol after activation of the adrenal gland induces *TPH2* gene expression and subsequently de novo 5-HT synthesis, which in turn inhibits

cortisol release from the adrenal cortex. In addition, animal and human studies have shown that different types of stressful events can increase 5-HT synthesis and release in specific brain regions such as the hippocampus (Lanfumey et al. 2008). Alterations in both stress and 5-HT systems might therefore interact to give rise to the neurophysiological phenotype seen in NR macaques.

A key component of the serotonergic neurotransmission is the transporter, which pumps back the released serotonin from the synapse into the presynaptic neuron and hence terminates its action on the postsynaptic neuron. The *serotonin transporter* gene (*5-HTT* or *SLC6A4*) is one of the most frequently studied candidate genes in psychiatry, because of a polymorphic region (called 5-HTTLPR) in its promoter, creating allele-specific expression of the transporter both in humans (Lesch et al. 1996) and in rhesus monkeys (Lesch et al. 1997; Bennett et al. 2002). In both species, the short allele (S) confers lower gene expression at the cellular level and elevated sensitivity to environmental influences at the behavioral level, whereas the long allele (L) confers higher mRNA expression and resiliency to adverse environmental effects.

A series of studies using the maternal deprivation model showed genetic effects of the rhesus macaques' 5-HTTLPR on a range of phenotypes, as well as G×E interactions involving rh5-HTTLPR and rearing conditions. These studies compared two genotype groups of the rh5-HTTLPR (the heterozygote LS vs the homozygote LL, due to the low frequency of the SS genotype) in their monkey samples. Bennett et al. (2002) observed that individuals with the LS genotype had lower 5-HIAA concentrations than homozygote LL individuals, but only among PR monkeys. At the behavioral level, rhesus infants with the LS genotype showed a higher level of distress compared to LL homozygotes in their first month of life, although there was also a main effect of rearing on this behavior, with PR infants displaying more distress (Champoux et al. 2002). Additionally, the LS genotype was associated with higher adrenocortical responses to separation stress paradigm in PR infants (Barr et al. 2004b), especially among PR females (Barr et al. 2004a). These studies demonstrate that carrying the 5-HTTLPR S allele makes individuals more sensitive to social adversities, resulting in altered HPA axis, serotonin metabolism, and further behavioral problems (Table 7.1). This early social life effect was also captured by a positron emission tomography (PET) study showing lower 5-HTT density in many brain regions including the hypothalamus, caudate, amygdala, and hippocampus in PR monkeys compared to MR (Ichise et al. 2006). Similar G×E effects have been reported in humans for the 5-HTTLPR genotype interacting with childhood abuse to increase risk for depression (reviewed by Caspi et al. 2003).

Other studies could compare the SS homozygotes to the other two rh5-HTTLPR genotypes in rhesus macaques living in large, outdoor colonies (only MR condition). The SS genotype showed increased stress reactivity (higher plasma cortisol levels) after separation or pharmacological challenge in 3-month-old infants (Sorenson et al. 2013) and higher hair cortisol level due to chronic stress in female adult monkeys (Qin et al. 2015).

Further G×E effects were observed within the *BDNF*, *CRH*, monoamine oxidase A, neuropeptide Y, and *TPH2* genes in rhesus macaques exposed to various forms of

Table 7.2 Interactive effects of genetic variations and rearing conditions. Overview of G×E interaction findings using the rhesus monkey (*Macaca mulatta*) maternal deprivation model, differences between *MR* mother reared, and *NR* nursery reared, *PR* peer reared, *SPR* surrogate-peer-reared conditions

Genetic variant	Differential rearing condition effect	Reference
5-HTTLPR	NR monkeys with LS genotype had lower orientation scores at 1 month PR monkeys with LS genotype had lower cortisol and higher ACTH responses to separation stress at 6 months PR monkeys with LS genotype had lower 5-HIAA at 2 years of age Adult PR and SPR male monkeys with LS genotype had higher level of aggressive responses In adult male hippocampus tissue MR monkeys with LS genotype had higher H3K4me3 enrichment at the 5-HTT gene	Champoux et al. 2002 Barr et al. 2004a Barr et al. 2004b Bennett et al. 2002 Schwandt et al. 2010 Lindell et al. 2012
BDNF 136 G/A Val46Met	PR monkeys with GG genotype had higher BDNF level at 1–2 months	Cirulli et al. 2010
CRH -248C/T SNP	PR T-allele carriers (CT+TT vs CC genotype) had higher cortisol and ACTH response to stress, decreased level of exploration at 6 months, and higher level of alcohol consumption at age 4–5 years	Barr et al. 2008
MAOA-LPR	Male MR monkeys with the low-activity allele had higher aggression scores	Newman et al. 2005
NPY -1002 T/G SNP	PR G-allele carriers (GT+GG vs TT genotype) had lower CSF NPY level, exhibited higher level of arousal during stress at 6 months, and consumed more alcohol at age 4–5 years	Lindell et al. 2010
OPRM1 77C/G SNP	OPRM1 77G allele exhibited stronger attachment to their mothers in baseline conditions and more intense distress responses to maternal separation 77G allele carriers exhibited lower levels of cortisol after maternal separation	Schwandt et al. 2011
TPH2 2015 A/C SNP	MR CC genotype (vs AA+AC) had lower level of aggressive threat PR CC genotype had lower plasma ACTH level	Chen et al. 2010

5-HTT serotonin transporter, *5-HTTLPR* serotonin transporter gene-linked polymorphic region, *ACTH* adrenocorticotropic hormone, *CSF* cerebrospinal fluid, *CRH* corticotropin-releasing hormone, *H3K4me3* histone 3 lysine 4 trimethylation, *MAOA-LPR* monoamine oxidase A length polymorphism, *NE* norepinephrine, *NGF* nerve growth factor, *MHPG* 3-methoxy-4-hydroxyphenylglycol, *TPH2* tryptophan hydroxylase 2 (brain specific), *NPY* neuropeptide Y, *OPRM1* opioid receptor μ1, *SNP* single nucleotide polymorphism

early life adversity (for details and references, see Table 7.2). Analyses considering interactions between diverse environments and more than one gene are obviously needed to understand the variety of phenotypes observed in humans and macaques. These initial studies give promising results on how genotype might interact with the environment in the development of human mental health. However, the mechanisms that might be responsible for mediating these interactions remain unclear; emerging evidence indicates that epigenetic mechanisms can play a major role.

Indeed, assessing histone mark, histone 3 lysine 4 trimethylation (H3K4me3), which indicates active promoters, Lindell et al. (2012) found that both rearing and rh5-HTTLPR genotype had significant effect on H3K4me3 level at the *5-HTT* gene promoter. Higher H3K4me3 was measured in hippocampal tissue of male rhesus macaques with the LS genotype compared to LL genotype, where higher levels were also observed in MR subjects compared to PR monkeys after controlling for age. In addition, infant macaques with the rh5-HTTLPR LS genotype were found to have higher mean DNA methylation (another epigenetic mark), at the *5-HTT* gene promoter and lower 5-HTT expression level in their blood cells (Kinnally et al. 2010). Interestingly, better quality of maternal care also predicted lower DNA methylation level at the *5-HTT* gene promoter in 4-month-old rhesus infants (all of them were MR), which in turn predicted better health outcomes in adulthood (Kinnally 2014).

In humans, such epigenetic mechanisms underlying G×E effects were also reported. For example, analyzing white blood cell DNA samples of patients with post-traumatic stress disorder (PTSD), allele-specific, trauma-dependent DNA demethylation of the *FKBP5* gene was found by Klengel et al. (2013). Allele-specific changes in DNA methylation following early life adversity have also been described by others (Kerkel et al. 2008; Gibbs et al. 2010; Meaburn et al. 2010; Zhang et al. 2010a; Gertz et al. 2011). More examples of epialleles in humans are described in detail in Sect. III of this book including the *FKBP5* gene. Taken together, these studies indicate that the DNA sequence also contains information on its methylation status, thereby offering a potential mechanism by which G×E interactions alter disease state later in life.

7.3 Epigenetic Effects Induced by Maternal Deprivation in Rhesus Macaques

7.3.1 DNA Methylation and Hydroxymethylation

This chapter focuses mainly on DNA modifications, but epigenetic components also include histone modifications, chromatin remodeling factors, and non-coding RNA. It is important to keep in mind that most studies investigate these marks independently, but in reality they interact to exert a combined modulation of gene transcription/translation conferring the cell their identities. Moreover, these marks not only regulate current gene expression profiles but could prime future events. The true nature of such relationships can only be assessed in a dynamic context, which requires parallel longitudinal and intergenerational studies that are difficult to conduct in humans.

DNA methylation is a covalent modification of the DNA at cytosine residues and is part of the regulation of tissue-specific gene expression during the life course. After cellular development, the methylation status of a gene is maintained by DNA methyltransferases (in particular DNMT1). However, environmental cues can

dynamically change the DNA methylation pattern and subsequently alter gene expression. Active (de novo) DNA methylation is catalyzed by DNMT3a and 3b (Okano et al. 1998). Active DNA demethylation can also occur via more complex DNA repair-based mechanisms (Morgan et al. 2004; Barreto et al. 2007; Rai et al. 2008; Ma et al. 2009a; Schmitz et al. 2009). In the most recently proposed model, 5-hydroxymethylcytosine serves as an intermediate modification of 5-methylcytosine, which is formed by ten-eleven translocation (TET) proteins (Kriaucionis and Heintz 2009; Tahiliani et al. 2009), and can lead to unmethylated cytosine replacement through nucleotide and/or base excision repair factors (Guo et al. 2011a, b). Hydroxymethylation might also serve as an intermediate step in passive demethylation occurring during DNA replication, since there is no existing mechanism for maintaining hydroxymethylation profiles during cell division. Alternatively, it could serve as a stable epigenetic mark, specifically in the brain (Li and Liu 2011), altering gene expression on its own by recruiting transcription regulators (Jin et al. 2010; Ficz et al. 2011; Mellen et al. 2012; Spruijt et al. 2013) or through binding of TET1 (Zhang et al. 2010a, b; Zhang and Meaney 2010) and/or TET3. See Fig. 7.2 for an overview of the life cycle of DNA methylation in mammalian cells. In addition to developmental processes, these epigenetic mechanisms have been shown to be involved in the immune response to pathogens as well as in neuronal activity and also underlie the mechanism of learning (Ma et al. 2009b; Guo et al. 2011a; Day et al. 2013; Garden 2013; Russ et al. 2013; Busslinger and Tarakhovsky 2014; Quintin et al. 2014). Hydroxymethylation was also recently shown to dynamically mediate behavioral adaptations (Li et al. 2014).

7.3.2 Early Timing Is Important for Long-Lasting Epigenetic Effects

Epigenetic regulation is a time-dependent and highly controlled process that contributes to cellular differentiation during gestation and postnatal development driven by innate developmental programs. In particular, DNA methylation is essential for mammalian development and cell specificity. Epigenetic profiles established in sperm and oocyte get extensively remodeled in the zygote and the embryo, a process that was also shown recently to be intimately linked to DNA hydroxymethylation (Guibert and Weber 2013). After birth, DNA modification profiles are still dynamically regulated through development. For example, DNMT3a methyltransferase, which is present in neuronal precursors and remains expressed in postmitotic neurons with the highest level of expression during the first three postnatal weeks (Feng et al. 2005), is believed to be critical for establishing brain DNA methylation profiles during perinatal development both in mice and humans (Lister et al. 2013). Similarly, brain DNA hydroxymethylation level progressively increases after birth and is suggested as crucial in the postnatal neurodevelopment (Szulwach et al. 2011; Lister et al. 2013). The importance of DNA methylation and hydroxymethylation in postnatal development is not limited to the brain but also

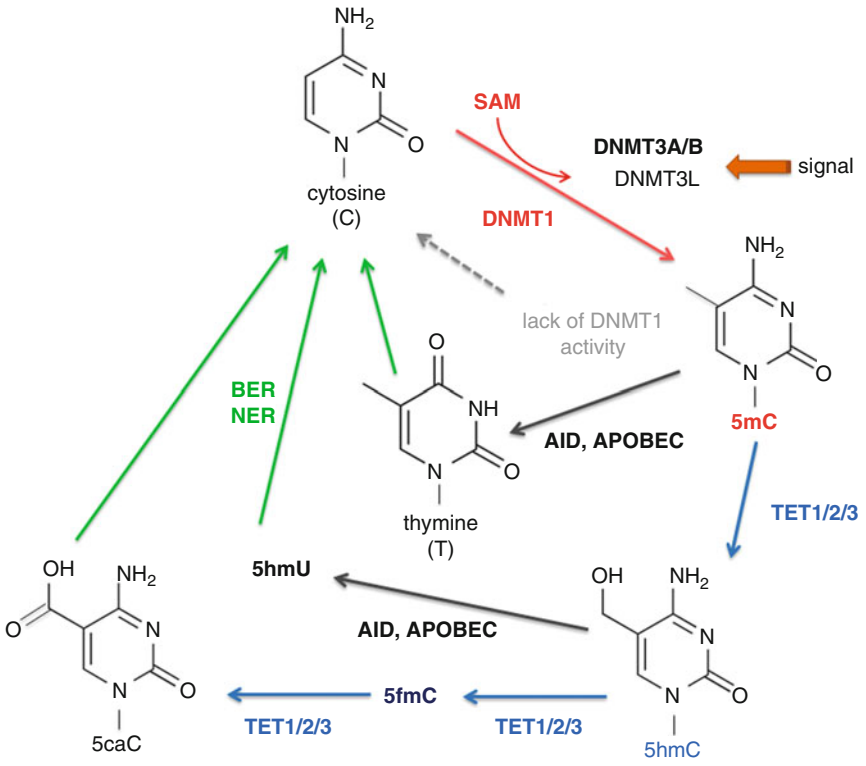


Fig. 7.2 The life cycle of DNA methylation in mammalian cells.
DNA methyltransferases (DNMT) methylate cytosines at the 5th carbon, using S-adenosylmethionine (SAM) and creating 5-methylcytosines (5mC). After replication, maintenance DNA methyltransferase 1 (DNMT1) methylates hemi-methylated DNA at CpG sites, while de novo methyltransferase DNMT3A or DNMT3B does not require hemi-methylated DNA to bind. The catalytic inactive member DNMT3L enhances the activity of de novo DNMTs due to its scaffolding function. Passive demethylation can occur in the absence of DNMT1 during consecutive replications. Active demethylation of 5mC is proposed through iterative oxidation by ten-eleven translocation proteins (TET1/2/3) producing 5-hydroxymethylcytosine (5hmC), then 5-formylcytosine (5fC), and lastly 5-carboxylcytosine (5caC). These cytosine analogues may impair binding and activity of the maintenance methylation machinery in mitotically active cells, leading to passive dilution of modified C. 5hmC can also be converted to 5-hydroxymethyluracil (5hmU) by cytidine deaminases (*AID* activation-induced cytidine deaminase, *APOBEC* apolipoprotein B mRNA editing enzyme, catalytic polypeptide). These cytidine deaminases can also convert 5mC to thymine (T). Finally, the mismatched bases are repaired by the base excision and/or nucleotide excision repair machinery (BER/NER)

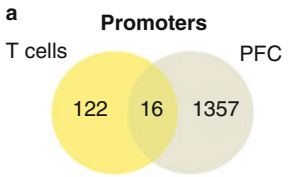
observed in other tissues, such as T cell development and differentiation (Tsagaratou et al. 2014; Rodriguez et al. 2015).

Extensive data established that the epigenome does not reach a fixed terminal pattern and can be affected by external stimuli conferring an environmental exposure-specific identity to DNA. This process can occur at different time points

in life. However, the epigenome is particularly plastic and sensitive to the environment at critical developmental stages when it undergoes extensive remodeling as described above. Therefore, epigenetic modifications occurring during gestation, in the early postnatal period, and during puberty can have major impacts on physiological functions throughout life. A typical example is the effect of maternal diet on one gene in the progeny of agouti mice that modulates one-carbon metabolism (Waterland and Jirtle 2003). Maternal diet supplementation with either phytoestrogen or bisphenol A increased or decreased the methylation level of the offspring retrotransposon that conferred a yellow coat color and later on an obese phenotype in the case of decreased methylation. Giving a methyl-rich diet to yellow and obese agouti mothers during pregnancy was sufficient to counteract this effect in the pups (Dolinoy et al. 2006, 2007a). In humans, the epigenetic effects observed by Klengel and colleagues were seen in PTSD patients that had experienced childhood trauma but not in those experiencing adult trauma. Similarly, DNA methylation changes that were reported in the progenitor hippocampal cells only occurred when the cells were treated during their proliferation and differentiation periods, suggesting critical periods during development for these changes to have long-lasting effects (Klengel et al. 2013). Additional studies in animals and humans have also shown that the social environment both prenatally and postnatally has long-lasting impact on adult physiology and psychopathology, with type and timing of stressors, as well as gender, as main moderating factors (Heim and Binder 2012; Provencal and Binder 2014a).

7.3.3 DNA Methylation and Hydroxymethylation Induced by Maternal Deprivation in Rhesus Macaques

To delineate the epigenetic response induced by maternal deprivation in the brain as well as in periphery, we previously examined genome-wide promoter methylation profiles from isolated T cells and from the prefrontal cortex (PFC) of adult rhesus males subjected to different rearing conditions (MR vs SPR). Using the method of methylated DNA immunoprecipitation (MeDIP) with comprehensive genome-wide microarray hybridization, we reported ~1300 genes with differential methylation in their promoter in the PFC and ~130 differentially methylated promoters in T cells at false discovery rate (FDR) < 0.2 (Provencal et al. 2012) (Fig. 7.3a). We also observed that similar DNA methylation differences tended to appear in clusters within chromosomes in both tissues. Indeed, by partitioning the genome into 500 kb regions, we identified 55 chromosomal regions in PFC and 68 regions in T cells showing enrichment for differentially methylated probes (FDR < 0.01) including a histone gene cluster in PFC and a zinc finger cluster in T cells (Fig. 7.3b). In addition, the differentially methylated genes were enriched in specific biological functions, such as neuronal development in PFC and immune response in T cells. When looking at the overlap between T cells and PFC changes, we found that 10 of these enriched



Location	Gene name	Description	More methylated		p-value	
			PFC	T	PFC	T
Chr2	**TRAK1**	Trafficking protein, kinesin binding 1	SPR	SPR	0.04	0.008
Chr6	**NR3C1**	Glucocorticoids receptor isoform 3	SPR	SPR	0.007	0.0007
Chr8	**TTC35**	Tetratricopeptide repeat domain 35	SPR	MR	0.02	0.03
Chr19	**ZNF724P**	Zinc finger protein 724 (pseudogene)	MR	SPR	0.009	5E-5
Chr20	**ZG16**	Zymogen granule protein 16 homolog	MR	SPR	0.003	0.05

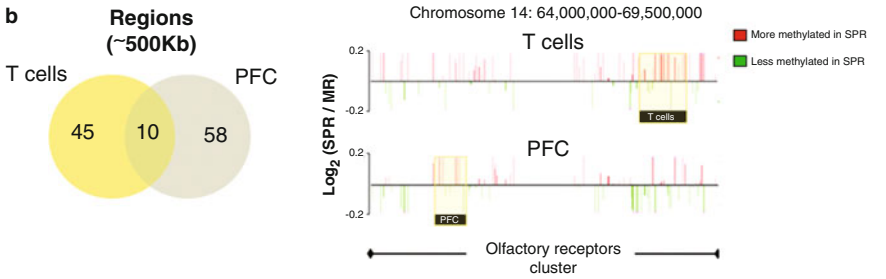


Fig. 7.3 Rearing-associated DNA methylation in adult male rhesus macaques' prefrontal cortex (PFC) and T cells. (**a**) Venn diagram depicting the number of gene promoters differentially methylated between rearing conditions in PFC and T cells as well as the overlap between both tissues (left panel). The table highlights five of these gene promoters where the exact same probe was found differentially methylated in both tissues (*right panel*). (**b**) Venn diagram depicting the number of differentially methylated 500 kb regions (DMRs) between rearing conditions in PFC and T cells as well as the overlap between both tissues (*left panel*). The figure on the right depicts an expanded view from the UCSC genome browser of the olfactory receptor cluster located on chromosome 14 found to be more methylated in SPR animals in both tissues. The average MeDIP probe fold differences (Log2) between MR and SPR are shown for T cells (top) and PFC (*bottom*). In *green* are promoters whose probes indicate lower methylation, and in *red* are those more methylated in the SPR animals. Highlighted in *yellow* are megabase large regions significantly differentially methylated between MR and SPR

chromosomal regions were shown to be differentially methylated in both tissues including the olfactory receptor cluster that was generally more methylated in SPR males (Fig. 7.3b). Moreover, 16 specific gene promoters showed rearing-associated methylation in both tissues. Interestingly, at the top of this list, the *NR3C1* gene was more methylated in the SPR group in both tissues (Fig. 7.3a). Taken together, these data suggest a broad impact of maternal rearing on DNA methylation level in the brain that could explain in part some of the phenotypic characteristic seen in SPR animals such as higher reactivity to stress and aggressive/impulsive behavior. To a less extent, DNA methylation alterations were also observed in T cells and suggest that immune-related genes are also affected by rearing. There is a growing body of evidence suggesting that the immune system is responsive to different types of early adversity such as social isolation and parental anxiety (Barreau et al. 2004; Danese

et al. 2007; Sloan et al. 2007; Powell et al. 2013; O'Connor et al. 2014). In addition, studies in rodents have shown that immune genes, such as cytokines, play an important role in brain development (Deverman and Patterson 2009). This supports the hypothesis that the response to early life adversity is system- and genome-wide and persists into adulthood.

As suspected, this molecular response to maternal separation is not limited to DNA methylation. For example, we reported DNA hydroxymethylation alterations in the PFC of differentially reared monkeys, at gene promoters that did not show altered DNA methylation patterns (Massart et al. 2014). These results were unexpected as we observed a strong correlation between DNA methylation and hydroxymethylation levels at promoters. However, these observations suggest that DNA hydroxymethylation might be considered as an additional epigenetic signal that mediates early life events. Changes in DNA hydroxymethylation but not methylation were detected at the promoters of monoaminergic genes, such as the *5-HTT*, the *DRD3 dopamine receptor*, or the *ADRA1 adrenoreceptor*. Hydroxymethylation modifications were also found at the transcriptional repressor *RE-1-silencing transcription factor* (*REST*) gene. Consequently, they might have affected neuronal development, creating different ratio of cortical cell subtypes. These cell type differences, if maintained through adulthood, can create substantial epigenetic differences between MR and SPR monkeys. Moreover, hydroxymethylation changes were not restricted to neuronal genes but were also observed at the astrocyte-specific isoform *ITPR2* (*inositol 1,4,5-trisphosphate receptor, type 2*). This result indicates that the brain's response to early adversity is not restricted to a specific cell type and points to the involvement of glial cells in behavioral disorders associated with maternal deprivation, as reported before in Fisher rats (Leventopoulos et al. 2007).

It is likely that at least part of the observed epigenetic changes induced by maternal deprivation are mediated by genetic variations. Indeed, the genetic background, such as specific polymorphisms in the *5-HTT*, *MAOA*, and *CRH* genes described above, is likely to influence the impact of the environment on the DNA methylation landscape. The experimental design used in the maternal deprivation model, in which each infant monkey is randomly assigned to one or another rearing condition, reduces the likelihood that group differences in DNA methylation are due to genetic polymorphisms. Nevertheless, the small number of animals tested, together with the absence of genome-wide genetic data, prevents to rule out the influence of genetic factors in these studies. Interestingly, a recent study identified such interactions using a genome-wide approach to analyze the genetic and in utero environmental influences on DNA methylation profiles in cord tissues from newborns (Teh et al. 2014). In this study, 25 % of the variable methylation sites was explained by genetic factors alone, and the rest (75 %) was explained best by an interaction between genetic and prenatal environmental factors. These data suggest that both genetic makeup and the environment are responsible for the establishment of the methylation profiles. It is important to note that the CpG sites analyzed by Teh et al. included only a small portion ($n = 1423$) of the total methylation sites in the genome with a very high variance cutoff for their variable regions (two continuous CpGs located within 1 kb with MAD score > 95th percentile) in a

particular sample. Therefore, it is probable that big differences in DNA methylation levels of neonatal tissues reflect genetic influence, whereas small-moderate differences can be solely due to stress-related prenatal factors, as it was shown for DNA methylation sites in neonatal cord blood samples associated with maternal depression (Non et al. 2014; Nemoda et al. 2015).

7.3.4 System-Wide Changes in DNA Methylation

The studies cited above support the hypothesis that epigenetic differences in response to social adversity are system-wide and are not limited to the brain. The DNA methylation differences observed in macaque peripheral blood cells might be mediated, in part, by stress regulators that are released into the blood circulation. The increased release of glucocorticoids observed following ELS, for example, could simultaneously target similar genes across diverse tissues because its receptors are present in most tissues. Subsequently, changes in DNA methylation could be induced in several tissues by these activated steroid hormone receptors in response to ELS (Szyf 2009, 2013; Provencal and Binder 2014b). For example, the *NR3C1* gene was one of the few genes to be less methylated in both the PFC and T cells in response to rearing in rhesus macaques (Fig. 7.3a) (Provencal et al. 2012). It might be that the change in DNA methylation is a consequence of increased *GR* transcription/activation and increased GR binding at these loci in both tissues, since we know that GR activation increases its own expression by a positive feedback loop. A great example supporting this hypothesis is the study by Klengel et al., described in detail in Sect. III.1 of this book, that identified a G×E interaction associated with demethylation of the *FKBP5* gene in blood cells. Interestingly, they found similar DNA methylation changes after glucocorticoid exposure in human progenitor hippocampal neuronal cells, suggesting that similar epigenetic mechanisms could be at play in response to an increased cortisol release in both tissues. Therefore, long-lasting effects of ELS are likely to be reflected in tissues outside of the brain and, if similar targeted genes or members of similar pathways are affected, could potentially serve as biomarkers for increased risk to developed psychiatric disorders, or cardiovascular and metabolic diseases.

The studies presented above report mainly tissue-specific effects on DNA methylation profiles in response to ELS with only limited and localized overlaps, but what are the expected correlations between brain and peripheral tissues in healthy subjects? Epigenetic profiles in specific loci might correlate highly across tissues and perhaps be more informative as peripheral biomarker of brain functions than less correlative regions. Indeed, recent studies have compared epigenome and transcriptome data of brain and blood samples to answer this question about intra- and interindividual variations in DNA methylation (reviewed by Tylee et al. 2013). One key conclusion is that between-tissue (intraindividual) variation in DNA methylation is much greater than interindividual differences within any given tissue; however, there are certain interindividual variations that are reflected across both

brain and blood samples (Davies et al. 2012). Interestingly, buccal cells might serve as a better biomarker than blood for epigenome-wide association studies in non-blood-based diseases due to a greater overlap of buccal differentially methylated regions (DMR) with other tissues at tissue-specific DMR compared to blood (Lowe et al. 2013). Furthermore, methylation patterns from four brain regions were more similar to buccal epithelial cells than to blood or saliva samples with low proportion of buccal cells (Smith et al. 2015). This overlap might be explained by the ectodermal origin of both epithelial and brain cells suggesting that these DNA methylation profiles are traces of early cell differentiation during development. Our preliminary data on DNA methylation changes in blood and buccal samples of 2-year-old SPR and MR male monkeys showed 1110 genes with significant methylation differences at their promoter in blood DNA samples and 416 differentially methylated promoters in buccal samples at FDR $\leq$ 0.05 (Nemoda et al. unpublished data). Interestingly, over 100 genes overlapped between our previous rearing-associated methylated genes from the PFC and either blood or buccal samples at FDR $\leq$ 0.2 (31 genes had significant DNA methylation changes in all three tissues). Taken together, these data indicate that easily accessible peripheral tissues, such as whole blood, saliva, or buccal cells, could be useful in clinical and epidemiological studies of complex neurobiological phenotypes.

7.4 Conclusion

The response to early environment is complex and involves multiple phenotypes and genomic loci in different physiological systems. Multiple studies have shown that suboptimal maternal care can induce broad transcriptional and epigenetic changes in the brain as well as in the immune system. Overall, the DNA methylation modifications are found organized and coordinated in clusters and loci, as described above in detail for the monkey model of maternal separation, strengthening the hypothesis that the response of the epigenome to early adversity is an adaptation to the actual environment. Hormonal transmission constitutes an attractive mechanism for a coordinated and systemic epigenetic response to the environment. Indeed, hormones are involved in the growth and regulation of many organ and systems such as the brain (Baud 2004; Auyeung et al. 2013; Moisiadis et al. 2014) and the immune system (Baschant and Tuckermann 2010; Pilipovic et al. 2012). Moreover, they exert powerful effects on the epigenome, the social environment affects their levels, and early endocrine perturbations have been associated with adult disease susceptibility (Dolinoy et al. 2007b; Murgatroyd et al. 2009; Klengel et al. 2013; Niwa et al. 2013; Moisiadis and Matthews 2014).

Most of the studies conducted to date have not addressed the questions of the persistence of the epigenetic changes induced by early adversity and if the modifications observed later in life are a result of these changes in epigenomic trajectories. A first hint to answer this very important question came from cross-fostering experiments and epigenetic drug treatments done by Weaver and colleagues. They were

able to show a direct relationship between maternal care, DNA methylation changes, and reversal of the phenotype supporting a causal relationship between DNA methylation and induced long-lasting phenotype variations (Weaver et al. 2005, 2006; Champagne et al. 2006). In our monkey model, we conducted a cross-sectional study on methylation profiles of maternally deprived and mother-reared monkeys during their first years of life and revealed 4924 and 2547 gene promoters differentially methylated at 14–30 days (infants) and 2 years (adolescent) after birth, respectively (Nieratschker et al. 2014). We observed a significant overlap of 1180 differentially methylated genes in both infant and adolescent monkeys. This result indicates that even though early adverse events induced numerous DNA methylation alterations, only 16 % of these persisted over the 2-year period indicating that most of these changes faded with time, whereas 18 % of total DNA modifications appeared 1 year after maternal separation occurred. This observation highlights the importance of conducting longitudinal studies in order to separate transient from long-lasting epigenetic effects of early life adversity. These can occur across the lifetime of an individual and are likely influenced by uncontrolled environmental and genetic factors. In addition, multi-omics data analysis combining multiple epigenetic marks as well as gene expression and genetic profiles are needed to understand these transient and long-lasting effects of early life environments on phenotypic variations. The causal links between these associations would also need to be assessed by conducting proper cross-fostering or adoption studies and intervention studies (pharmacological and psychological) including replication in an independent sample. Since most of these studies will be difficult to assess entirely in humans, socially interactive animal models, such as monkeys, are essential to decipher and hopefully prevent these long-lasting alterations induced by early life adversity.

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Chapter 8
Stress, Transposons, and the Brain Epigenome

Richard G. Hunter

Abstract Long thought to be transcriptionally silent junk, transposable elements (TEs) are emerging as sources of functional elements in mammalian genomes due to the introduction of modern deep sequencing techniques. They have begun to attract the attention of neuroscientists due to the observation that the brain appears to be a privileged environment for transposon activity. In the brain, TEs show active transposition and frequently interact with the epigenetic machinery during development and in response to environmental inputs like stress. Barbara McClintock, the discoverer of TEs, long asserted that these elements were an important part of the genomic control apparatus, particularly in response to stress to the organism. Recent work has shown that this observation was a prescient one, as stress shows the capacity to alter the activity of these elements in the brain, in some cases with both adaptive and pathogenic consequences. TEs have been recently implicated in a number of mental disorders including Rett syndrome, posttraumatic stress disorder (PTSD), and schizophrenia. TE-derived regulatory RNA may comprise one of the largest single classes of functional elements in our genome, a discovery which will have a profound effect on how gene-environment interactions are understood within the context of the nervous system and beyond.

Keywords Retrotransposon • Glucocorticoid • Steroid • Noncoding RNA • Mental disorders • Neurodegeneration

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8.1 Introduction

Stress plays a significant role in a number of mental and physical disorders and can have lasting effects upon the brain and behavior. Within the brain, stress has been shown to effect neural plasticity in a variety of ways with clear behavioral consequences for the stressed organism. How stress can have such persistent effects, some of which span generations, has been the subject of intensive research much of which has focused on epigenetic mechanisms in recent years. Epigenetics offers a clear explanatory framework for how environmental inputs like stress can have long-lasting, even heritable effects without inducing changes in the structure of the genome itself. However, as research going back to Barbara McClintock's groundbreaking work in the 1940s and 1950s has shown, the genome itself is more dynamic than the classical understanding of genetics would have it. McClintock discovered a class of mobile genomic elements, which she called "controlling elements" which could reshuffle parts of the maize genome with heritable effects on plant phenotype. Most importantly for the present discussion, she noted that these effects were most often induced by stresses or "shocks" to the plant (McClintock 1951, 1984). Transposable elements (TEs) or transposons have since been found in the genomes of almost every taxon of life, including mammals where they make up roughly 50 % of the genome on average. While long regarded as "junk" DNA, these elements have recently attracted the attention of neuroscientists who have shown that they are highly active in the brain and that they appear to be under some degree of epigenetic control, findings which have substantial implications for our understanding of brain function and brain disorders (Hunter et al. 2013, 2014; Reilly et al. 2013; Erwin et al. 2014; Griffiths and Hunter 2014).

8.2 Transposons

Transposable elements (TEs) are a diverse class of genomic elements that share the capacity to move themselves from one genomic location to another. They have also played a significant role in genome evolution, genome structure, and cell fate determination. More recently it has become evident that many of them are actively transcribed and that some transposon-derived RNAs, like the Xist (X-inactive specific transcript) lncRNA, which governs X-chromosomal inactivation, play significant functional roles in both development and disease (Pontier and Gribnau 2011). Arguments asserting that these elements are merely parasitic or junk are based in part on Ohno's assertion that a maximum of 20,000 coding loci were possible based on estimates of the rate of deleterious mutations available at the time (Ohno 1972). However, these rates were likely overestimated and were, moreover, based on the assumption that protein sequence was the major molecular target of selection. It is now evident that most of the genome is actively transcribed and that much of this transcribed RNA may be the product of RNA genes, rather than the protein-coding genes circumscribed by the central dogma (Consortium 2012; Mouse et al. 2012; Fu 2014).

Transposons are divided taxonomically into DNA transposons and RNA transposons or retrotransposons (RTs); the former use a mechanism similar to cutting

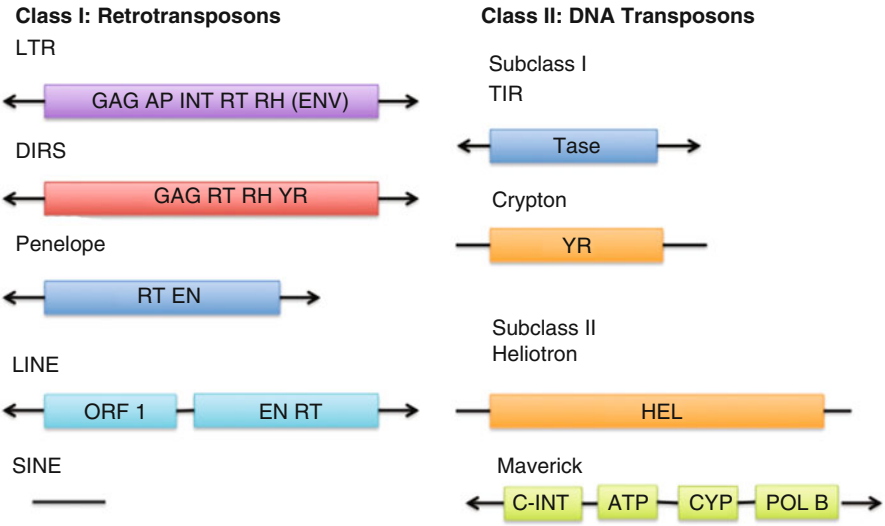

Fig. 8.1 Classification of transposable elements (TEs). TEs are subdivided into class I or II based on the requirement for an RNA intermediate in the transposition process. Class I retrotransposons are further subdivided into the LTR (long terminal repeat/endogenous retrovirus), DIRS (*Dictyostelium* intermediate repeat sequence), Penelope, LINE (long interspersed nuclear element), and SINE (short interspersed nuclear element) superfamilies based on their internal architecture and terminal repeat sequences. DNA or class II transposons are divided into two subclasses (1 and 2). Subclass 1 contains the TIR (terminal inverted repeat) and Crypton superfamilies, and subclass 2 contains the Heliotron and Maverick superfamilies. Protein-coding domains: *AP* aspartic protease, *ATP* packaging ATPase, *C-INT* C-integrase, *CYP* cysteine protease, *EN* endonuclease, *ENV* envelope protein, *GAG* capsid protein, *HEL* helicase, *INT* integrase, *ORF* open reading frame, function undetermined, *pol B* DNA polymerase B, *RH* RNAse H, *RT* reverse transcriptase, *YR* tyrosine recombinase. This classification is a simplification of the system proposed by (Wicker et al. 2007)

and pasting in text editing, while the latter typically copy and paste using RNA intermediates (See Fig. 8.1). Both varieties can be either autonomous or nonautonomous depending on whether or not they encode their own transposition machinery or not (Wicker et al. 2007; Levin and Moran 2011). Due to the relatively low fidelity of most transposases, many nonautonomous TEs are incomplete or missense duplications of autonomous TEs. Both major taxa are further subdivided into subclasses, orders, and so on down the line through superfamilies and families to individual TEs. RTs constitute the single largest class of genomic elements in mammalian genomes and are subdivided into five subclasses which include long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), long terminal repeat or LTR transposons, DIRS (*Dictyostelium* intermediate repeat sequence), and Penelope-like RTs. LINEs, SINEs, and LTRs represent roughly 18 %, 12 %, and 8 % of the human genome, respectively, while DNA transposons and other varieties of RTs represent less than 3 % (Cordaux and Batzer 2009). LINEs, in their intact form, are approximately 6 kb in length and encode one or two open reading frames (ORFs), one of which codes for a reverse transcriptase and an endonuclease (Wicker et al. 2007). In the most common human

LINE, L1, the first ORF also codes for a nucleic acid-binding protein whose function is not completely understood (Goodier et al. 2013). Of the roughly 500,000 L1 elements in the human genome, less than 100 appear to be intact and capable of causing transposition (Brouha et al. 2003). The remainder depends on these few for the chance to mobilize, as do all of the SINE elements. These elements, of which *Alu* and SVA are most common in humans, are too short, at less than 200 bases in length, to code for their transposition machinery. LTR retrotransposons include endogenous retroviruses that have invaded genomes in cycles over millions of years. Like the LINEs, most are inactive though some retain the capacity to transpose, and a few, like the IAP elements in rodents, are still capable of forming functional virus particles (Sharif et al. 2013).

While the prevailing view has been that transposons are parasitic, and it is inarguable that some of them certainly have a parasitic origin, it has been asserted since McClintock's discovery of these "controlling elements" that they play a useful role in the genomes that contain them (McClintock 1951, 1984). It has long been evident that they have played a significant role in genome evolution (Kazazian 2004). The LTR class alone is responsible for an estimated 10 % of all spontaneous mutations in the mouse genome (Maksakova et al. 2006). Transposition is a source of exon shuffling and recombination, significant means in the creation of new genes and genomic elements (Moran et al. 1999; Abrusan and Krambeck 2006). With the advent of large-scale sequencing, it has become apparent that many of these nonprotein-coding elements are actively transcribed, and some such as Xist and HOTAIR (HOX antisense intergenic RNA) have been shown to have roles in normal physiology as well as disease (Rinn et al. 2007). Indeed, the ENCODE project now identifies many transposon-derived long noncoding RNAs (lncRNAs) as genes comprising as much as 20 % of the functional genome (Harrow et al. 2012). It remains controversial what fraction of lncRNAs are transposon derived versus having an origin as protein-coding genes or other nongenic elements of the genome. However, it is clear that a substantial fraction of vertebrate lncRNAs (65–85 %) contain TE-derived sequences and that relatively little sequence is contributed by protein-coding sequences (less than 0.5 %) or UTRs (less than 30 %) (Kelley and Rinn 2012; Kapusta et al. 2013). TEs and lncRNAs are also more sequence specific in their expression than protein-coding genes, suggesting that they could play a significant role in the determination of the extraordinary variety of neuronal phenotypes (Cabili et al. 2011; Gage and Muotri 2012; Kelley and Rinn 2012; Reilly et al. 2013). Transposons also show much higher levels of interindividual variation than protein-coding genes (see below), which means they may contribute to some of the wide variance in susceptibility and resilience seen in many complex diseases.

8.3 Transposons and the Brain

Neuroscientists have turned progressively more attention toward the role of transposable elements in the brain as the technology of next-generation sequencing (NGS) has made it possible to observe them in detail. Before the NGS era, transposons had

been connected to a variety of human disorders, such as hemophilia and cancer (Kazazian et al. 1988; Solyom et al. 2012), as well as in the proper function of the immune system where the V(D)J system responsible for antibody diversity is derived from TEs (Kapitonov and Jurka 2005) and in the telomeres that are maintained by a TE-derived enzyme, telomerase (Nakamura and Cech 1998). Telomeres also utilize many of the same types of noncoding RNA/heterochromatin interactions that obtain in TE silencing. Interest in brain TEs was low until the observation that L1 LINE transposition occurs in mammalian neurons, particularly during development and neurogenesis, and that the brain is, therefore, more of a genetic mosaic than had been thought. It has been theorized that this mosaicism provides beneficial neuronal diversity in an analogous way to the use of transposon-derived reshuffling methods present in the immune system (Muotri et al. 2005; Baillie et al. 2011; Gage and Muotri 2012). This process is regionally specific, as neurogenesis in the adult brain is restricted to the hippocampus and rostral migratory stream, whereas transposition rates in the cortex appear to be relatively low (Evrony et al. 2012). In the hippocampus, the rate of transposition is sensitive to environmental interventions like stress and exercise (Muotri et al. 2009) and to be controlled in part by elements of the molecular epigenetic machinery like MeCP2 (methyl CpG binding protein 2) (Muotri et al. 2010). It is thought that, by promoting greater fitness in the organism, TEs increase the likelihood of their propagation, which is the foundation of symbiosis (Reilly et al. 2013; Erwin et al. 2014). Indeed, it has been argued in a number of contexts that TEs exist in a symbiotic relationship with host genomes though the point remains unresolved (Ryan 2004; Upton et al. 2011; Hunter et al. 2013, 2014).

TEs have certainly contributed to the evolution of gene promoters, particularly with regard to steroid response elements, the binding sites where steroid hormone receptors interact with DNA (Cotnoir-White et al. 2011). The brain, like other steroidogenic tissues (such as the placenta and gonads), seems to be a privileged site for TE activity (Pillai and Chuma 2012; Hunter et al. 2014; Ross et al. 2014). In humans and other primates, Alu SINEs have contributed response elements for vitamin D, progesterone, and glucocorticoid receptors (Gombart et al. 2009; Jacobsen et al. 2009). Steroid receptors themselves have been implicated in chromosomal rearrangements similar to those produced by transposons and may act in concert with TEs to do so (Lin et al. 2009; Holzman 2010). This suggests that steroids may act to activate some TEs, as indeed they do in the few studies that have sought to examine this phenomenon. Androgens increase LINE expression, and the L1 LINE ORF-1 protein seems to interact directly with the androgen receptor itself in prostate cancer cells to activate AR gene targets (Morales et al. 2002; Lu et al. 2013). TEs represent a source of both genomic and phenotypic variance at the cellular level that steroids may interact with to shape and canalize across the course of development and within the emergency life stage represented by stress. Indeed, it recently has been suggested that TEs may act in this way in the sexual differentiation of the brain as well as in gonadal development (McCarthy et al. 2015). Glucocorticoid stress hormones have been shown to upregulate the expression of SINE elements in the liver as well (Sun and Frankel 1986). Steroids are in part responsible for orchestrating responses to environmental conditions across the organism, so their potential

to mobilize TEs as sources of genomic restructuring, as well as potential RNA diversity, is an exciting area deserving further exploration, nowhere less so than with regard to stress and the stress response.

8.4 Transposons and the Epigenome

Molecular epigenetic mechanisms include histone modification, DNA methylation, and noncoding RNA. Given that many ncRNAs are transposon derived, the epigenetic role of these elements is established. However, this is not the limit of transposon-epigenome interactions. Transposons are important determinants of heterochromatin domains, and their RNAs are directly involved in the regulation of chromatin state (Bodega and Orlando 2014). Perhaps the best-known example of the interaction of transposable elements with the epigenome is the agouti locus in the mouse. The DNA methylation status of this locus controls coat color in the mice that carry it, and the core methylation site is derived from an IAP ERV/LTR retrotransposon (Michaud et al. 1994; Morgan et al. 1999). IAP elements, unlike the majority of ERVs, retain some capacity for producing infectious virus particles and are highly active in the mouse genome (they are thought to be responsible for as much as 5 % of the mutations) (Maksakova et al. 2008; Ribet et al. 2008). Because IAP elements are incompletely tamed, they are under tight epigenetic control, particularly during conditions of stress. In addition to DNA methylation, these elements also seem to be marked with the repressive histone H3 lysine 9 trimethylation (H3K9me3), which appears to be the major brake on their expression as deletion of the repressor, TRIM28/KAP1, results in IAP overexpression. TRIM28 (tripartite motif-containing 28) is responsible for maintenance of H3K9me3 as well as DNA methylation at IAP and other ERV/LTR loci (Rowe et al. 2010, 2013; Hunter et al. 2012). With regard to heterochromatic control of TEs, it appears that both facultative and constitutive heterochromatic marks, histone H3 lysine 27 trimethylation (H3K27me3) and H3K9me3, are involved, though they appear to target different classes (Day et al. 2010). Similarly, L1 retrotransposition is under epigenetic control in the brain via the actions of histone deacetylase and the methylated DNA-binding protein MeCP2. In patients with Rett syndrome, a neurodevelopmental disorder caused by mutations in the MeCP2 gene, L1 retrotransposition rates appear to be higher (Muotri et al. 2010). This is also the case in schizophrenia, though it is less clear what the mechanism might be for increase retrotransposition in the disorder (Bundo et al. 2014) These TE control mechanisms have been co-opted to form the basis of the developmental silencing of areas to the genome in order to specify cell fate (Bodega and Orlando 2014). Disruption of developmentally appropriate epigenetic silencing by an expansion of non-transposon repetitive elements is the pathogenic mechanism in fragile X syndrome (Mirkin and Mirkin 2014), and similar processes may be at work in Huntington's disease as well (Evans-Galea et al. 2013), which suggests that this mechanism may obtain in other neurodevelopmental disorders and neurodegenerative disorders.

The X chromosome imprinting machinery uses the lncRNA Xist to guide the heterochromatin-mediated inactivation of one X chromosome in female mammals. Xist is derived from both the exons of a decayed protein-coding gene and a number of TEs likely cobbled together in a process like exon shuffling (Elisaphenko et al. 2008). Xist is transcribed from the inactive X chromosome and serves as a scaffold for the facultative heterochromatin machinery, notably the polycomb repressive complex, PRC2, which trimethylates H3K27. Xist is itself regulated by the lncRNA Tsix, which is Xist antisense transcribed by the active X chromosome and Xist. Tsix also appears to bind to the PRC2 complex via the same repeat motif utilized by Xist suggesting that it may inhibit inactivation of the active X chromosome both by acting as an interfering RNA and as a competitor for PRC2 binding (reviewed in (Pontier and Gribnau 2011)). Tsix also appears to bind to the PRC2 complex using the same repeat motif utilized by Xist, and this appears to be the first identified case of a more general phenomenon where lncRNA serves to recruit chromatin-modifying enzyme complexes. Other TE-derived lncRNAs have been shown to interact with different chromatin-modifying complexes, such as CoREST (REST corepressor) and trithorax proteins (Khalil et al. 2009; Schuettengruber et al. 2011; Casa and Gabellini 2012). Both of the aforementioned complexes play significant roles in development and cell fate determination. Many transcribed lncRNAs (many TE derived) were discovered by scanning for a chromatin sequence characteristic of actively transcribed protein-coding genes (H3K4–H3K36 trimethylation), providing evidence that these elements share the same sort of epigenetic regulation as classically defined genes (Khalil et al. 2009).

Recruitment of repressive complexes to transposable elements makes sense if they are purely parasitic, and indeed there is evidence that repressive histone H3 lysine 9 and H3 lysine 27 trimethylation are associated with distinct classes of transposable elements (Day et al. 2010; Rowe and Trono 2011; Hunter et al. 2012). However, as the example of actively marked lncRNA genes above makes plain, some of these elements are targeted by activating epigenetic writers. It has been proposed that TEs are a source of functional domains for the creation of lncRNA genes and that these domains enable the variety of interactions between nucleic acids and proteins of which noncoding RNAs are capable (Johnson and Guigo 2014). This supports the idea that TE-derived genomic elements are important for the structure and function of the epigenome itself.

8.5 Transposons and Stress

TEs are more likely to transpose under conditions of stress, as their discoverer noted (McClintock 1951, 1984). Endogenous retroviruses, which comprise a substantial portion of mammalian RTs, are also more likely to replicate under conditions of stress to the host (Cho et al. 2008). Psychostimulants, which produce many of the same endocrine and neurochemical effects as a stressor, have been shown to alter both TE transcription and transposition (Maze et al. 2011; Okudaira et al. 2014). Due to the

danger that active viruses and genome reshuffling pose, organisms have developed a number of mechanisms to ensure that these elements are well controlled. Many of the epigenetic silencing mechanisms that are responsible for chromatin structure and the regulation of cell fate appear to have evolved in part to control potentially parasitic TEs (Fedoroff 2012). This static level of TE control via heterochromatinization has long been observed and provided some of the evidence for the view that TEs were transcriptionally inactive, a view we now know to be incomplete at best. The relationship between TEs and the epigenetic mechanisms involved in their control is not as simple as the suppression of parasitism, as we have explained above. In most if not all organisms examined, TEs and the mechanisms of genome regulation have evolved together, co-opting these elements in order to increase their utility to the organism. This seems particularly true with regard to increasing the capacity of the organism to adapt to environmental conditions, particularly aversive ones. At the evolutionary level, TEs provide a source of on-demand capacity for genome reshuffling and gene creation via duplication, deletion, and insertion events. Examples of the adaptive effects of TEs on genomes span the range from bacterial insertion sequences to the TE-derived enhancer sequences in the mammalian pro-opiomelanocortin gene and include a number of cases where TE activity has increased in response to specific environmental conditions such as temperature or rainfall (Casacuberta and Gonzalez 2013). Some TEs may confer stress sensitivity to nearby genes, as is the case with the rice DNA transposon, *mPING* (Naito et al. 2009). We have proposed elsewhere that TEs might represent a store of potentially useful genetic code in complex organisms which has less access to the horizontal gene transfer networks present in prokaryotes and unicellular eukaryotes (Hunter et al. 2014).

But rapid adaptation to population level stress is not the only space in which transposons and the stress response interact. Many transposons are acutely activated by stress signals (Casacuberta and Gonzalez 2013), and the yeast retrotransposons Ty5 completely alters its activity and transposition targets in response to environmental stress (Dai et al. 2007). Another example is CDT-1 (chromatin licensing and DNA replication factor 1), which was originally identified as a desiccation tolerance gene in plants but later shown to be a TE. Consistent with the emerging theme of co-opting TEs into noncoding RNA genes, CDT-1 produces RNA, which helps orchestrate the desiccation tolerance response (Hilbricht et al. 2008). Another significant example is the upregulation of SINE elements in response to heat shock in both mice and humans (*Alu* and *SINE B2*, respectively). Heat shock response is one of a number of evolutionarily conserved mechanisms of cellular stress response present in eukaryotes. In both man and mouse, these TEs are actively transcribed by RNA pol II in response to heat shock, and their RNA binds to RNA pol II to block transcription of protein-coding genes (Mariner et al. 2008; Yakovchuk et al. 2009). Suppression of transcription is an important part of the heat shock response, which is dedicated in part to the prevention of potentially cytotoxic protein misfolding (Verghese et al. 2012). More than any other example, this work clearly demonstrates the adaptive utility of TEs in the stress response of individual organisms.

In this context, the recent findings that stress can rapidly, over the course of an hour or so, downregulate the transcription of numerous RTs in a stress-sensitive brain

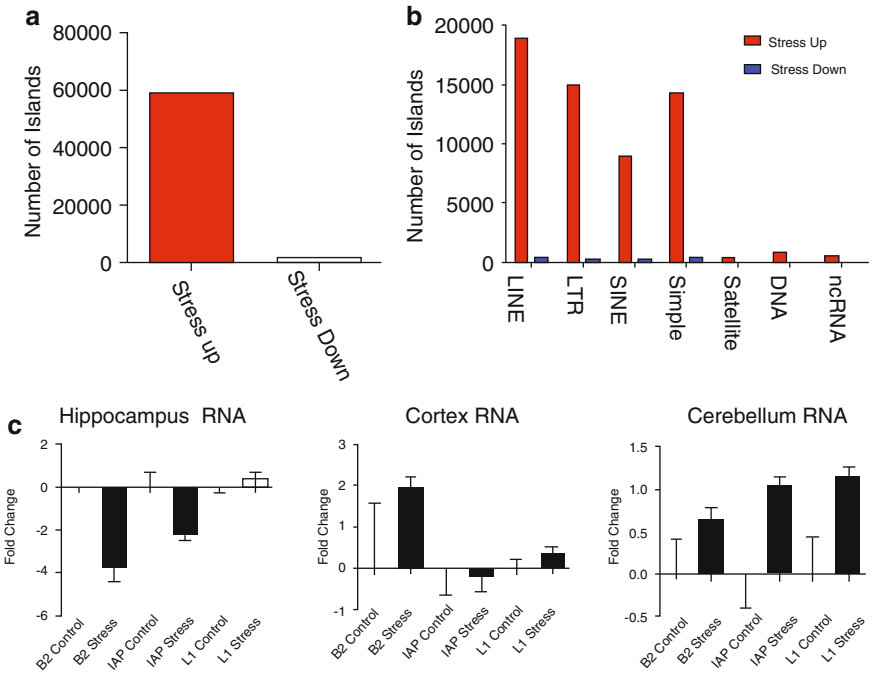

Fig. 8.2 Stress-induced epigenetic regulation of retrotransposons in the hippocampus. (**a**) shows the total number of genomic elements (arbitrary) that showed increased (stress up, *red*) or decreased (stress down, *blue*) H3K9me3 in rat hippocampus after an acute stress exposure. (**b**) breaks down the changes in H3K9me3 shown in A, by repeat class; retrotransposons showed pronounced increases in H3K9me3. (**c**) shows the regulation TE RNA after stress of a few individual examples of SINE (B2), LTR (IAP), and LINE (L1) retrotransposons. Downregulation is specific to the hippocampus, while in the cerebellum, where the H3K9me3 response was not observed, TE expression was increased (*$-p<0.05$, **$p<0.005$) (Adapted from Hunter et al. (2012))

region are particularly striking. In the rat hippocampus, via rapid increases in heterochromatic histone 3 trimethylation at lysine 9 (H3K9me3), a single immobilization stress represses the transcription of numerous retrotransposons at as many as 60,000 different genomic loci (See Fig. 8.2) (Hunter et al. 2009, 2012). Interestingly, this downregulation, which included B2 SINE elements, was hippocampus specific, with most tissues observed showing no significant change in expression, while one area, the cerebellum, showed an increase in TE RNA expression after acute stress (Hunter et al. 2012). Rapid downregulation of RT expression would, of course, be superfluous if they were silent; however, they are not. In contrast, hippocampal H3K9me3 levels decline with repeated stress, while amygdala expression of at least some LINEs is increased in more chronic models of stress and alcoholism (Ponomarev et al. 2010, 2012). The observations that B2 elements are reduced in expression after an acute psychological stress while they are increased after heat shock suggest that these elements and others like them are subject to fine-scale regulation in response to environmental conditions, rather like genes, and like genes they have a functional role to play

in the cells that express them. The available evidence suggests that many if not most of these elements are transcribed at some level in the brain and other tissues. In fact, their expression is highly tissue and cell type specific (Faulkner et al. 2009; Reilly et al. 2013). Given this set of facts, it is unsurprising that they are stress regulated.

Stress and stress-related disorders show large variance across individuals in terms of their impact. PTSD, for example, affects roughly 7 % of the population, while a majority of the population is exposed to trauma at some point in their lifetime (Kessler et al. 1995; Breslau et al. 1998), raising significant questions about what makes some individuals vulnerable and others resilient. TEs show much wider variability across individuals than protein-coding genes, and most of us have at least one that may be "private" and may not exist in another member of our species (Iskow et al. 2010); thus, they may represent a means to understand both interindividual variance in stress resilience and the "missing heritability" observed in many stress-related psychiatric disorders (Manolio et al. 2009; Crow 2011). Indeed, dysregulation of the brain and peripheral transposon expression has been observed in animal models of PTSD and alcoholism as well as in human subjects suffering from the same disorders (Ponomarev et al. 2010, 2012; Rusiecki et al. 2012). Ectopic overexpression of TEs, like chronic stress, may contribute to brain aging and neurodegenerative disorders. In *Drosophila*, as in the mammalian brain, TEs contribute to brain mosaicism (Perrat et al. 2013), and they are under the control of epigenetic mechanisms including the dsRNA processing machinery including Argonaute proteins and TDP-43 (transactive response DNA-binding protein 43 kDa). In aging flies, transposon expression expands producing a "transposon storm" which contributes to age-associated neuronal deficits (Li et al. 2013; Reilly et al. 2013). A similar process has been implicated in human frontotemporal dementia (Li et al. 2012). Loss of control of Alu SINE RNA expression due to declining dicer1 expression has been shown to be a mechanism in certain varieties of age-related macular degeneration, suggesting that decline in the ability to control the expression of TE RNA may be a common culprit in brain aging (Kaneko et al. 2011; Tarallo et al. 2012). Stress also contributes to brain aging and to biological aging in general; chronic stress reduces telomere length, for example (Mora et al. 2012; Shalev et al. 2013). The work showing that stress is also involved in the control, or lack of control, of TE expression suggests that the two may be mechanistically connected with regard to brain aging and neurodegeneration. Much remains to be explored about how exactly TEs play a role in both normal physiology and pathology. It will also be necessary to disentangle and clarify the differences between transposons and the ncRNA genes, which are to a large extent composed of TEs if such a distinction can be clearly made.

8.6 Conclusions

Transposons are ubiquitous but poorly understood genomic elements, which represent a potentially enormous store of information and regulatory mechanisms. While research into the pathogenic potential of transposable elements is several decades old, much remains to be discovered. The beneficial functions of these elements are more

controversial, and for the most part much more recently described. Though the discoverer of TEs, Barbara McClintock, argued from the outset that their role was an adaptive rather than a parasitic one, that view was overridden until recently by eminences such as Francis Crick and Susumu Ohno, who argued that they were junk. Evidence from large-scale sequencing projects such as ENCODE has helped to change this view, particularly by demonstrating that most of the genome is transcribed at one level or another. It is clear from the examples given here that these elements are not without function nor are they without the potential to induce pathology.

With regard to the effects of stress upon the brain, these elements are particularly exciting as a potential means of explaining the wide individual variation in stress response and in stress-related diseases from PTSD to type II diabetes. That being said, much work needs to be done to establish a mechanistic relationship between these elements, the epigenome and the lasting effects of stress upon the brain and behavior. With some exceptions, most of the work done to date is correlational in nature, and we do not have an integrated understanding of all of the potential interactions and mechanisms in which TEs might be involved. In particular it is important to work toward understanding the mechanisms by which TE expression is regulated and to what physiologic signals they respond. Stress plays a significant role, so too might sex steroids, what other systems might be involved? It is probable that signals of cellular distress, such as elevated reactive oxygen species or caspase activation might act as switches, but as yet the question has not been addressed. Given that TEs represent a tenfold larger fraction of our genetic material than the protein-coding genes that have been at the center of molecular biological attention for the better part of a century, this dearth of knowledge should not be surprising. The level of complexity this implies means that we will have to develop not only new bioinformatics tools but new ways of thinking about cellular, epigenome, and genome function. However, the potential to explain phenomena like missing heredity (or complex causality) in the complex diseases that now represent most of the disease burden in the developed world makes this daunting task clearly worthwhile.

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Part II
Epigenetics and Sexual Differentiation

Chapter 9
Neuroepigenetics of Sexual Differentiation of Brain and Behavior

Margaret M. McCarthy, Sara L. Stockman, and Bridget M. Nugent

Abstract Sexual differentiation of the brain occurs during a developmental sensitive period under the influence of gonadal steroids. As understanding of the brain has increased in sophistication, so too has the awareness that the enduring consequences of hormonally mediated differentiation are not understood. Epigenetics offers a means by which changes in gene expression can be established and maintained or reactivated by the adult steroid milieu. While still in the earliest stages of discovery, some principles are emerging including that masculinization of brain and behavior involves an escape from repression by DNA methylation and that not all epigenetic changes can be temporally tied to hormone exposure. Steroid modulation of enzymes regulating DNA methylation and histone modifications create the divergence in epigenetic patterns in some regions of male and female brains. RNA-Seq highlights diversity in both gene expression and transcriptional variation due to specific promoter usage and/or splicing. Challenges abound, but the future is bright for unraveling many mysteries of brain development in both males and females.

Keywords Estrogen • Sex differences • Hypothalamus • Preoptic area • Methylation • MicroRNA • Epigenetics

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There has been a renaissance in epigenetic studies in recent years due largely to discoveries of both the surprising degree of modulation in the nervous system and novel sources of regulation such as the noncoding RNAs. Day and Sweatt coined the term neuroepigenetics to conventionalize the many unique aspects of epigenetic regulation in the nervous system (Day and Sweatt 2010). Renewed interest in this form of gene regulation is further increased by the ever widening realization that events that occur during development are enduringly imprinted onto the genome, thereby impacting adult responses to a variety of stimuli. These include metabolism, stress axis activation, immunological profiles, and a range of social and emotional behaviors. Moreover, even in the mature brain, highly salient events leave their mark via changes to the epigenome. Understanding how both early and later life experiences alter gene expression of the brain in an enduring way is a current goal toward which much progress is being made. The sexual differentiation of the brain is a process that seems inherently designed for epigenetic regulation as it involves developmental exposure to hormones which "permanently" organize neuronal development toward a male or female phenotype so that adult physiology and behavior maximizes reproductive success. The intent of this review is to focus on what we have learned so far about the role of epigenetics in sexual differentiation and highlight the most promising areas for future exploration.

9.1 Methylation of CpG

Addition of a methyl group to the 5′ carbon on cytosine residues located proximal to guanines (CpG - the p stands for phosphate to indicate the C-G are not pair-bonded but adjacent to each other) is the most frequent direct modification of the DNA that is above the genome. CpGs occur in nature less frequently than expected by chance, and when they do, the most common state is methylation. DNA methylation is accomplished by a family of DNA cytosine-5-methyltransferases (DNMTs) that are each attributed with a unique function. DNMT1 exhibits a preference for hemimethylated DNA and thus is recognized as a maintenance methyltransferase (Yoder et al. 1997; Pradhan et al. 1999). De novo methylation is thought to be catalyzed by DNMT3a and DNMT3b (Hsieh 1994; Okano et al. 1999). These two enzymes are distinguishable by temporal patterns of expression in which DNMT3b is limited to embryonic stem cells, whereas DNMT3a is expressed both embryonically and postnatally (Watanabe et al. 2006). It has been proposed that DNMT3b coordinates de novo methylation embryonically, while DNMT3a is responsible for postnatal de novo methylation and this would include that occurring during the period of sexual differentiation. However, recent evidence invokes caution in the simplistic interpretation of the DNMTs as maintenance versus de novo. DNMT3a often only methylates one strand of DNA, producing hemimethylation, therefore requiring DNMT1 for complete methylation. DNMT1 also appears to have considerable de novo methylation capability independent of DNMT3a and DNMT3b (reviewed in (Jeltsch and Jurkowska 2014).

The canonical view of DNA methylation is that it serves as a physical blockade that disrupts protein binding and gene transcription. CpG methylation-induced repression of gene expression is even more effectively accomplished by the binding of methyl-binding proteins (MBDs) to methylated DNA and subsequent recruitment of nuclear corepressor and histone deacetylase repressor complexes (Nan et al. 1998). Methyl-CpG-binding protein 2 (MeCP2) was the first MBD to be characterized (Meehan et al. 1989), but there are now known to be a family of these proteins which can interfere with the formation of a transcriptional complex.

The simplicity of DNA methylation inhibiting transcription is intuitively appealing, but as with most things in biology, there is much more to it than that. Indeed, it now appears that DNA methylation can be an echo of past transcriptional activity or that it may be for purposes of regulation of differential promoter usage and splice variants. A majority of DNA methylation is found outside the context of so-called CpG islands and is instead in the intergenic regions where it acts to repress activation of retrotransposons as opposed to actual transcription. Thus, we are still in the midst of understanding both the dynamic and regulatory profiles of DNA methylation, including that non-CpG methylation and 5-hydroxymethylation may also be important signaling components (reviewed in McCarthy and Rissman 2014).

9.2 Sexual Differentiation of Brain and Behavior

Sex determination begins with chromosome complement, which in mammals is XX and XY. The tightly orchestrated temporal expression of the Sry gene of the Y chromosome is an obligate switch for the gonadal anlage to develop into a testis (Goodfellow and Lovell-Badge 1993). In the absence of Sry, gonadal development proceeds along a predetermined pathway and differentiates into an ovary. The sexual differentiation of the brain is inextricably tied to the differentiation of the gonads via the production of steroid hormones. Gonadal differentiation occurs remarkably early in fetal development, during the first weeks of gestation in humans and days 10–13 in rodents (O'Shaughnessy 2014). By the second trimester in humans and the last few days of gestation in rodents, the fetal testis is producing large quantities of androgens which circulate in the bloodstream and ultimate gain access to the brain. Neurons express the receptors for androgens, as well as for estrogens and the enzyme that converts androgens to estrogens, aromatase. In rodents, it is frequently the estradiol derived from testosterone and synthesized in the brain that initiates the cellular cascades that differentiate the brain toward a masculinize phenotype (reviewed in (McCarthy 2008)). In developing females, the ovaries remain quiescent until sometime after birth, and while there may be late steroidogenesis that contributes to feminization of the brain (Bakker and Baum 2008), it is essential that the female brain not be exposed to high levels of androgens or estrogens during the sensitive period. If exogenous hormone is administered to females during this time, there is a permanent propulsion of the developmental trajectory of the brain toward masculine end points. Likewise, if the male is deprived of high levels of exposure to

gonadal steroids during the sensitive window, masculinization will never be achieved (McCarthy et al. 2009).

Although the process of sexual differentiation of the brain is restricted to a narrow developmental sensitive window, there are an impressive number of changes across the brain that will impact the physiology and behavior of the adult. These include large-scale changes in the size of specific regions as well as highly localized shifts in synaptic patterning and neuronal and astrocyte morphology. The neurochemical identity of some neurons is impacted by hormone action, and rates of both neurogenesis and cell death are profoundly altered during the sensitive period. Hormonal modulation of neural circuitry and intrinsic neuronal properties mediate diverse physiological processes like control of anterior pituitary secretions (gonadotropins, growth hormone), stress responsiveness, metabolism, and so on. Behaviors across the spectrum from reproductive copulatory and parenting responses to fear and anxiety, food preferences, problem-solving strategies, play behavior, and pair-bonding are all found to differ in males and females under baseline or challenge conditions (Simerly 2002; Morris et al. 2004; Forger 2009; Lenz and McCarthy 2010).

Much progress has been made in elucidating the cellular mechanisms coopted by steroids to exert such broad and diverse influences. But the question of how these early developmental effects endure into adulthood has generally not been considered. Sensitive periods have a beginning and an end. In this instance, these parameters were operationally defined as the onset being when gonadal steroid production by the fetal male testis begins (~E16 and E18 in mice and rats, respectively) and the offset being the point at which exogenous hormone treatment is no longer effective at converting the female brain into the male phenotype. The precise timing of the offset can vary for different end points, i.e., size of a specific brain nucleus versus control of the LH surge, but interestingly in each case the end of the sensitive period appears to be abrupt, not gradual. This suggests it is not just a simple developmental process that gradually erodes the capability for change but that instead there is a defining biological process that closes the door of sensitivity.

The study of sexual differentiation of the brain is actually long running, having begun in the late 1950s (Phoenix et al. 1959). The first reports of robust sex differences in the mammalian brain that could be tied to early hormone exposure were in the early 1970s and involved the discovery that a small collection of Nissl dense cells in a subregion of the preoptic area was substantially larger in male rats compared to females (Gorski et al. 1980). Ultimately, it was determined that this sex difference was the product of differential cell death; specifically, the cells in this area died in neonatal females during the sensitive period because they lacked the trophic effects of estrogens that the male received due to aromatization of testicular androgens (Davis et al. 1996). This same principle was found to apply to other volumetric sex differences, and thus it was easy to conclude that the reason this sex difference endured into adulthood was due to the simple fact that death is permanent. Other sex differences in the size of neuronal projections (Simerly 2000) or the

morphology of dendrites (Mong et al. 1999) and frequency of synapses (Amateau and McCarthy 2002) were also considered structural, and so terms such as neuronal "wiring" or "architecture" which were coded for by a developmental "blueprint" led to the general perception that organizational actions of steroids produce permanent change by altering structure, and then function follows from structure.

Multiple advances both in the broader field of neuroscience and specifically in neuroendocrinology of the brain have forced a rethinking of the view that sexual differentiation of the brain is permanent because structural changes cannot be reversed. First is that we now know the mature brain continues to make new neurons in a few specialized niches (Gould and Gross 2002; Abrous et al. 2005; Epp et al. 2009) and there is ongoing cell production outside these zones as well, including in and around the sexually dimorphic nucleus (SDN) of the preoptic area at puberty (Ahmed et al. 2008). We also now appreciate that the formation of synapses is a highly dynamic process and that axons and dendrites are capable of extending and retracting. Thus, the end points we thought were permanent really aren't. In retrospect, this is consistent with reports that females could be induced to behave like males as adults if given a specific hormonal regime such as very long or large doses of androgens or that males could be induced to behave like females if also provided with exogenous hormones in a particularly restricted regime (Olster and Blaustein 1989). These reports were often ignored (including by these authors), as there was no viable explanation and the effects seemed "pharmacological." But perhaps they were instead telling us something that indeed the process is reversible and would require understanding how sexual differentiation effects endure and, more importantly, what closes the sensitive period.

9.3 Epigenetics of Sexual Differentiation of the Preoptic Area (POA) and Male Sexual Behavior

There are two general approaches to the study of epigenetics of sexual differentiation. First is to simply ask, is there a difference between males and females in a particular epigenetic mark? Thus, one would measure the amount of DNA methylation either broadly or at a specific locus, or one might quantify the frequency of a particular histone modification and perhaps follow with CHIP-Seq to find out what genes are associated with that modification. The second approach is to either interfere with or induce the placement of epigenetic marks during the critical period. Both the methylation of DNA and changes to the histones are accomplished by enzymes, making them readily druggable targets. As discussed above, there are a limited number of DNMTs, and so these can also be genetically deleted in mouse models. The very large number of enzymes involved in histone acetylation, deacetylation, and methylation has made genetic approaches less tractable, but pan-enzyme inhibitors are effective tools. Pharmacological manipulation of both the DNA-modifying and histone-modifying enzymes has provided insights into how epigenetics mediates sex differences in brain and behavior.

9.3.1 Females Have Higher Rates of DNMT Activity and More DNA Methylation than Males

We recently explored the role of DNA methylation in the sexual differentiation of the preoptic area and adult copulatory behavior. This brain region is essential for proper expression of male sexual behavior. Lesions of the preoptic area cause males to lose all interest in sex, and stimulating it causes males to lose interest in anything but sex (Hull and Dominguez 2007). Masculinization of the synaptic profile of preoptic area dendrites during the developmental sensitive period is essential to the ability of testosterone to activate male copulatory behavior in adulthood (Amateau and McCarthy 2002, 2004). Males have twice the density of dendritic spine synapses as females, and this pattern endures from the first few day of life to adulthood, leading us to ask, how is this cellular memory maintained? We began by looking for sex differences in the amount of DNMT enzymes and DNA methylation (Nugent et al. 2015). Quantification of mRNA and protein for all three DNMT enzymes in the neonatal rat preoptic area indicated no sex differences across the first week of life, but an enzyme activity assay revealed that the enzymes were more active in the female and could be suppressed by estradiol treatment. This sex difference was evident in the first few days but gone after 4 days of postnatal life. Importantly, the amount of methylation on the DNA paralleled the DNMT activity such that females had higher levels than males, and again this was reduced by treatment with a masculinizing dose of estradiol. Genome-wide bisulfite sequencing determined that it was the most highly methylated CpG sites that were overrepresented in the female genome compared to the male. This suggested that a set of genes were being actively epigenetically suppressed in the female and that these genes may be essential for masculinization (Fig. 9.1). Confirmation of this was established by treating newborn females with inhibitors of DNMT activity which induces demethylation of the DNA. Females treated with either zebularine or RG108, two specific DNMT inhibitors with different mechanisms of action, and then raised to adulthood and provided with male levels of testosterone displayed male-typical copulatory behaviors and a male-typical pattern of excitatory synapses in the POA. In a parallel fashion, female mice in which the DNMT3a enzyme was genetically ablated only in the POA were also highly masculinized. This provided important confirmation that it was the DNMT3a enzyme that specifically mediates masculinization in this brain region.

9.3.2 The End of the Sensitive Period Is Mediated by DNA Methylation

As noted above, sexual differentiation occurs during a sensitive period of development, and once that period passes, there is no longer the plasticity required to differentiate the brain as male or female. The end of the sensitive period had been considered to be due to simple maturational events that precluded ongoing plasticity, but there were unexplained reports of exceptions to the rule. The observation that DNA methylation maintained brain feminization raised the interesting specter that if the methylation was removed, plasticity could be reinstated. Toward this end, we

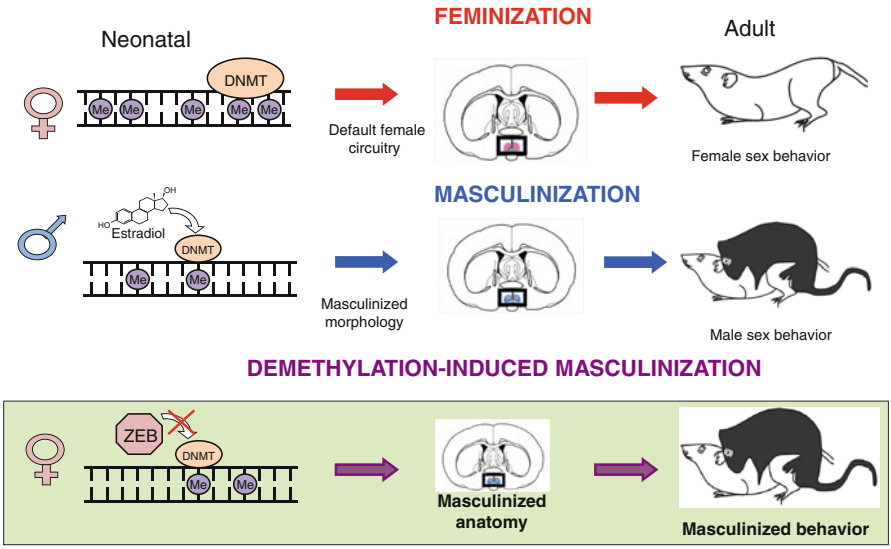

Fig. 9.1 *Masculinization of copulatory behavior requires demethylation of DNA in the preoptic.* During the neonatal sensitive period, there is higher DNMT activity in the preoptic area of females than males, and this is paralleled by more DNA methylation at CpG sites in females. Estradiol, which is aromatized from testicularly derived testosterone in males, inhibits DNMT activity and reduces DNA methylation. Treatment of females with a pharmacological inhibitor of DNMT activity reduces DNA methylation in the preoptic area and induces masculinization of brain and behavior (Nugent et al. 2015)

treated females with a DNMT inhibitor outside of the sensitive period and found that indeed they were masculinized both in brain and behavior. Careful examination of the time course of DNMT activity revealed that the sex difference was only evident during the sensitive period. More importantly, treating females with a masculinizing dose of estradiol outside the sensitive period not only did not masculinize them, it also failed to reduce DNMT activity as it had done during the sensitive period. From these findings, we conclude that the closing of the sensitive window is secondary to the loss of estradiol-induced reductions in DNMT activity and thus the inability to demethylate the DNA, as normally occurs in males (Fig. 9.2). Moreover, the feminization of the brain, while still the default, must be actively maintained by ongoing DNA methylation by DNMT3a. This may have implications for variation in human sexual behavior, including partner preference and gender identity that are not easily explainable by variation in prenatal hormone exposure.

9.3.3 Steroids Act on DNMT Activity to Regulate Gene Expression

An additional implication of this work is an expansion of the means by which steroids regulate gene transcription. Steroids bind to nuclear receptors that act as transcription factors either by binding directly to sequence-specific hormone response elements on

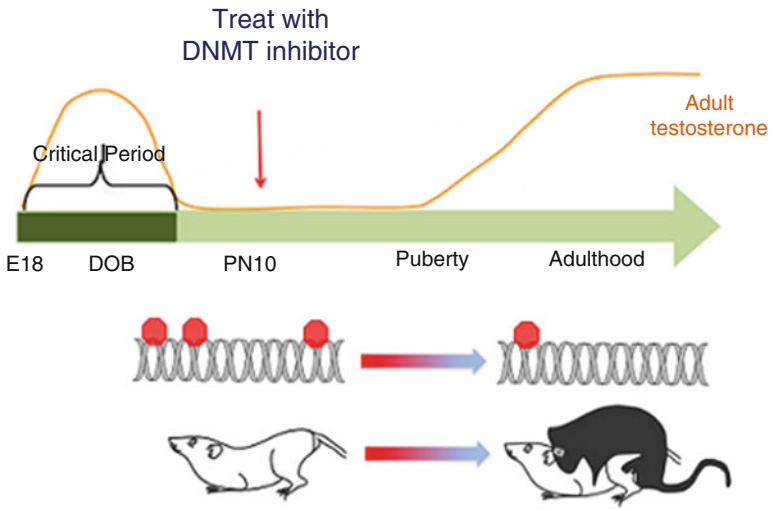

Fig. 9.2 *The end of the sensitive period is determined by regulation of DNA methylation.* The close of the sensitive period for sexual differentiation of the brain is operationally defined as the developmental time point at which exogenous hormone treatment of females is no longer effective at inducing masculinization. Treatment of females with a pharmacological inhibitor of DNMT, or Cre-mediated deletion of DNMT3a, reduced DNA methylation and induced masculinization outside of the sensitive period. Treatment of females with a masculinizing dose of estradiol at this same time did not inhibit DNMT activity and did not induce masculinization, demonstrating that the close of the sensitive period is mediated by DNA methylation (Nugent et al. 2015)

the DNA or by associating with other transcription factors such as c-fos (Beato and Klug 2000). The actions of steroids have been greatly expanded in recent years with the detection of their presence in the membrane and the ability to directly activate signal transduction pathways associated with GPRs and transmembrane kinases (Kelly et al. 1999; Abraham et al. 2004; Revankar et al. 2005). However, our findings invoke a new means for steroid action in the developing POA, the emancipation of genes by a reduction in DNA methylation (Fig. 9.3). The relationship between DNA methylation and gene expression is not simple however, and we found multiple genes that actually decreased in expression following DNA demethylation, further adding to the complexity of transcriptional regulation in the context of sexual differentiation.

9.3.4 Candidate Masculinization Genes Identified by RNA-Seq

Pinpointing the specific genes directing the masculinization process is far more challenging than establishing that DNA methylation is involved. RNA-Seq quantification of mRNA from the POAs of males and females with and without DNMT inhibition provided a profile of gene expression under normal and demethylation conditions. A surprisingly small number of genes were overall expressed at different levels in

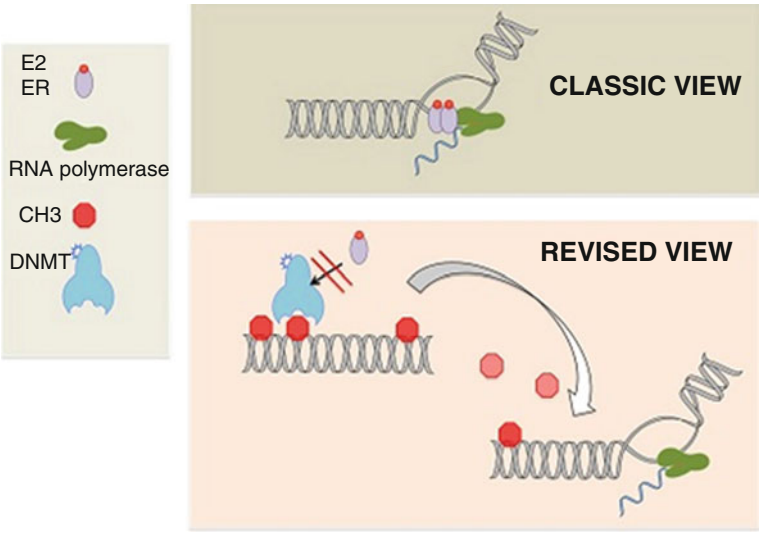

Fig. 9.3 *New view of steroid action during sexual differentiation of the preoptic area.* The classic view of steroid hormone effects on gene transcription invokes the binding of estradiol (E2) to its cognate receptor (ER) which translocates to the nucleus and binds to the DNA at a hormone response element. Masculinization of the brain by E2 would therefore involve transcription of specific genes. However, the ability of E2 to inhibit DNMT activity and reduce DNA methylation provides a new view of steroid action that is above the genome yet still profoundly impacts gene expression

males and females, and, even more surprising, they were evenly distributed between the sexes. RNA-Seq is a particularly powerful technique because of its ability to detect isoforms, and many more of these were found to differ in male and female POA. Those genes that overlap in males and females treated with a DNMT inhibitor are candidate masculinization genes, and here a very small number were identified (Fig. 9.4), with even fewer being confirmed by PCR. Ironically, the most reliably detected and confirmed gene was Cyp91a, the aromatase enzyme which converts testosterone to estradiol in the brain and has long been known to be essential for masculinization. The promoter region of the Cyp19a gene was found to be particularly highly methylated compared to other detected genes, suggesting this enzyme is poised for epigenetic regulation and that local steroidogenesis may be an important regulatory step (Fig. 9.5).

9.3.5 Chromatin Modifications Are Also Implicated in Sexual Differentiation of the POA

Histone modifications in the same brain region, the POA, are also implicated in regulation of aromatase and masculinization of copulatory behavior. Here, a combination of the two approaches was effective in elucidating that the promoter regions for the

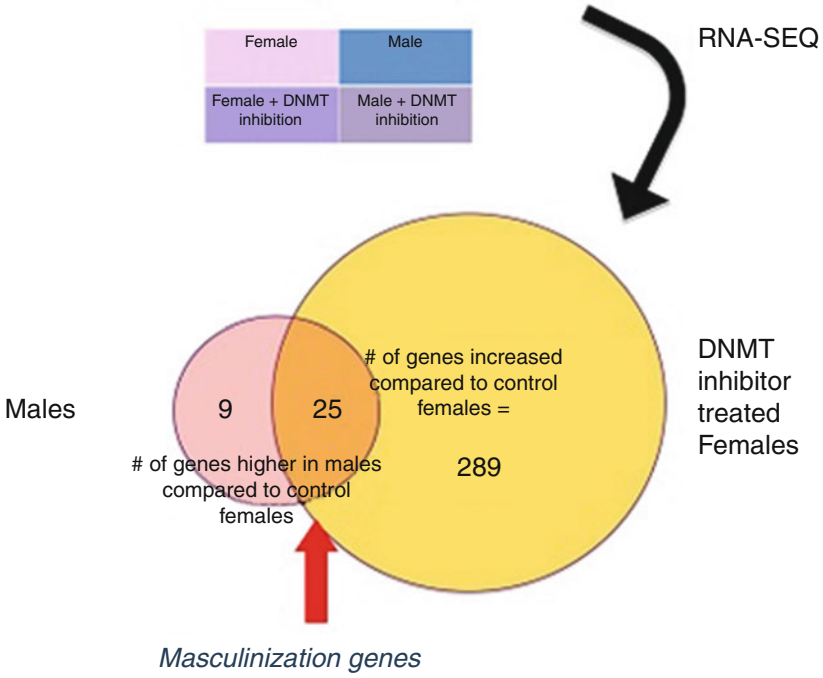

Fig. 9.4 *Candidate masculinization genes identified by RNA-Seq.* Newborn male and female pups were treated with an inhibitor of DNMT activity or a vehicle control and the resulting transcriptome of the preoptic area assessed by RNA-Seq. The number of genes that were expressed at different levels in males and females was relatively few, ~70, with 34 of those more highly expressed in males. When females were treated with the DNMT inhibitor, many more genes increased (314), and 25 of those were among the 34 that had been found to be higher in males. Thus, these are considered candidate genes for masculinization

ERalpha and aromatase genes were both more highly acetylated in males and that stimulating deacetylation via treatment with trichostatin A during the sensitive period for sexual differentiation impaired masculinization (Matsuda et al. 2011). Moreover, while the overall amounts of HDAC3 and HDAC4 did not differ in males and females, treatment of males with antisense oligonucleotides against histone deacetylase enzyme impaired the masculinization process. Thus, more than one enzyme is essential for the normal process of steroid-induced masculinization of sexual behavior.

A focused attack on one histone mark, H3K4me3, detected 248 genes that differed in the relative levels of this mark on the genome of males and females in the POA, and >70 % were higher in females. This particular piece of the histone code is associated with the edges of promoters and thereby closely aligned with transcription start sites. Although the sex difference in most of the genes could not be replicated by PCR at baseline, this does not mean that expression regulation is not different in males and females, and the observation that most of the genes are associated with synaptic function increases the potential regulatory impact of this difference (Shen et al. 2014).

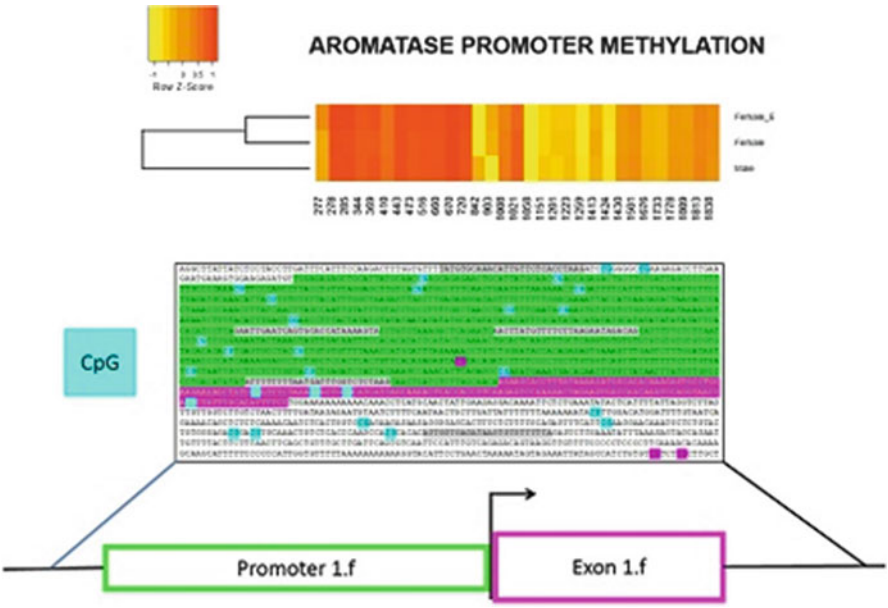

Fig. 9.5 *The promoter of the masculinization candidate gene Cyp19 is heavily methylated in the preoptic area*. Identified among the candidate masculinization genes was the well-known player, aromatase (Cyp19). Detailed examination of the promoter for this gene detected an unusually high level of methylation compared to other candidate genes and reveals a potential new source of regulation of this critical gene

9.3.6 Effects of Steroids on DNA Methylation May Be Temporally Delayed

The challenge of mapping the entire methylome can be partially circumvented with reduced representation bisulfite sequencing (RRBS) which selects for only those regions in the genome that are highly methylated and includes the majority of promoters. By limiting the substrate for sequencing, a greater number of comparisons can be made between biological conditions, an important advantage when studying sex differences. Exploiting this fact, Vilain and colleagues made the surprising observation that early testosterone treatment of neonatal females generated very modest gene expression changes on the short term but exerted a robust influence on adult expression profile (Ghahramani et al. 2014). A similar delayed epigenetic response was observed in a more circumscribed analysis of the promoter regions for ERa, ERb, and the progesterone receptor (PR) in several brain regions. Here, specific CpG methylations were dimorphic in males and females at some time points but not others, with marks disappearing and appearing across development (Nugent et al. 2010). How this epigenetic echo is generated is unknown but alerts us to how much we still have to discover in the realm of hormonal modulation of the methylome, as well as what are the implications for behavior.

9.4 Epigenetics of Sexual Differentiation of Social Play Behavior

The amygdala is critical for social and emotional processing. It has been shown to be important in regulation of many behaviors including social interactions, fear responding, and anxiety. The amygdala is a sexually differentiated brain region, and these differences emerge as a result of developmental organization. Males and females are known to differ with respect to volume, proliferation, and protein expression in particular nuclei of the amygdala (Mizukami et al. 1983; Hines et al. 1992; Krebs-Kraft et al. 2010). The amygdala also plays a role in coordinating well-recognized sex differences in juvenile social play behavior (Meaney and Stewart 1981; Auger and Olesen 2009). However, both the origins and nature of the sex differences in the amygdala are not fully understood.

9.4.1 DNMTs Are Expressed at Different Levels in Male and Female Amygdala

Evidence supports a role for epigenetic modification, especially through alteration in DNA methylation, in shaping sexual differentiation in the developing amygdala. Analyses of methyltransferases DNMT1 and DNMT3a at two time points in postnatal development revealed that females express significantly more DNMT3a in the amygdala as newborns but by 10 days of age the sex difference is no longer apparent. Note that the time course here is the same for the POA, but that here the amount of the enzyme differed in males and females, a fact not true for the POA. Newborn females treated with estradiol or dihydrotestosterone expressed significantly less DNMT3a compared to control-treated females indicating that both testosterone metabolites are responsible for the decrease in DNMT3a levels (Kolodkin and Auger 2011). No differences in DNMT1 expression were found at either age.

9.4.2 DNA Methyl-Binding Proteins and Corepressors Are Also Different in Males and Females

Given the lower levels of DNMT3a in the amygdala of males and the understanding that methyl-binding proteins like MeCP2 are recruited following methylation, it follows that males would also express less MeCP2 than females. Indeed, amygdalar expression of MeCP2 is lower in newborn males than females. This difference, as with DNMT3a levels, was temporary and did not persist to PN10. No differences were noted at either time point in another methyl-binding domain protein, MBD2, suggesting tightly controlled sex differences in expression (Kurian

et al. 2007). Protein binding to methylated DNA can facilitate additional binding of nuclear corepressor complexes, of which nuclear receptor corepressor (NCoR) was the first identified (Horlein et al. 1995). NCoR is recognized to act as a corepressor molecule for androgen and estrogen receptors (Lavinsky et al. 1998; Cheng et al. 2002; Yoon and Wong 2006). However, NCoR is also known to repress gene expression independently through interactions with MDPs like MeCP2 (Cukier et al. 2008). In accordance with the reductions in MeCP2 expression in the developing male amygdala, males express lower levels of the corepressor NCoR in the amygdala at birth. These differences were likely established by differences in steroid hormone exposure, as treatment of females with estradiol reduced expression of NCoR to levels similar to males (Jessen et al. 2010). Together, these data, which identify sex differences throughout the establishment and actions of DNA methylation, underscore the existence of sex differences in epigenetic regulation and implicate this mechanism in modulation of sexually differentiated behavior in the amygdala.

9.4.3 Sexual Differentiation of Social Play Is Regulated by DNA Methylation

Juvenile social play behavior is one of the earliest sexually differentiated behaviors to emerge during development. That male rats engage in higher frequencies of play compared to females has been known for close to 30 years (Olioff and Stewart 1978). Several neurodevelopmental disorders display deficits and delays in acquisition of social behavior. Rett syndrome is among these disorders and is known to be primarily caused by a genetic mutation in MeCP2 (Amir et al. 1999; Wan et al. 1999). More subtle reductions in MeCP2 expression may be associated with male-biased phenotypically similar disorders (Shibayama et al. 2004; Nagarajan et al. 2006). Recognizing an association between reduced MeCP2 expression and male-biased neurodevelopmental disorders, investigators explored the effect of brief MeCP2 disruption within the developing amygdala on development of social behavior in male and female rats. Transient reduction of MeCP2 expression significantly reduced male play behavior to levels typical of females. The same MeCP2 disruption in females produced no alteration in play behavior. Effects of MeCP2 transient reduction were specific to play behavior and did not effect juvenile sociability or adult anxiety (Kurian et al. 2008), reflecting a specificity in behavioral regulation. And an additional but complicated role for DNA methylation is introduced by the observation that siRNA-mediated reduction in GADD45, a key enzyme involved in demethylation via base excision repair, dramatically increases juvenile play by females (Kigar et al. 2015). Moreover, reducing GADD45 increases MeCP2, further supporting the view that this DNA methyl-binding protein regulates gene expression relevant to the sexual differentiation of social play and, more interestingly, raises the possibility that the MeCP2 gene itself may be epigenetically regulated differently in males and females.

9.4.4 *Vasopressin Is a Target of Epigenetic Regulation of Social Play*

The neurohypophyseal hormone arginine vasopressin (AVP), which is also widely ramified throughout the brain, mediates development of social play behavior by acting within the bed nucleus of the stria terminalis (BST) and the centromedial amygdala (CMA) (Veenema and Neumann 2009). Males express two to three times more AVP in both of these brain regions (De Vries and al-Shamma 1990). This sex difference is also organized during the critical postnatal period by the actions of steroid hormones on estrogen receptors (ERs) and androgen receptors (ARs) (Han and De Vries 2003). AVP was thus investigated as a candidate gene for mediating reductions in MeCP2 expression on disruptions in male juvenile social play behavior, potentially through actions on ER or AR. Transient reduction in MeCP2 reduced AVP in the amygdala of males 2 weeks after infusion. Males had decreased amygdalar AR following MeCP2 disruption, but no effect on ER was detected. Alterations in AVP cell number persisted into adulthood, but AR cell number showed no lasting effect of transient MeCP2 reduction (Forbes-Lorman et al. 2012). These results are somewhat unexpected given the traditional role of MeCP2 in repression of gene transcription through the binding of promoter regions, as has been demonstrated for AVP in the hypothalamus (Murgatroyd et al. 2009). However, MeCP2 increases gene transcription through interaction with cAMP response element-binding protein1 (CREB1) (Chahrour et al. 2008). Males maintain elevated CREB-binding protein (CBP) and phosphorylated CREB in several brain areas, and in these regions cAMP pathways have been implicated in organization of sex differences (Auger et al. 2001, 2002). Elevated CREB activity in males may facilitate MeCP2-induced gene transcription to organize sex differences in play behavior through mechanisms involving AR and AVP (Forbes-Lorman et al. 2012). Therefore, disruption of MeCP2 would actually cause a reduction in expression of these genes and create subsequent aberration in male juvenile social play behavior. Identification of lasting consequences on AVP as a result of transient reduction in MeCP2 enhances understanding of the role of MeCP2 in regulation of socially relevant behaviors through organization of neuronal phenotypes (Fig. 9.6).

9.4.5 *Maternal Care Sex Biases Also Impact Epigenetics in the Amygdala*

While AR is recognized as a critical regulator of sex differences within the amygdala, many of the effects of testosterone occur through aromatization to estradiol and subsequent binding to estrogen receptors (Naftolin et al. 1975). Throughout development, ERα predominates and exerts an extensive role in sexual differentiation (Kudwa et al. 2006). ERα expression is modulated by maternal care through modifications in DNA methylation patterns within the promoter region (Champagne

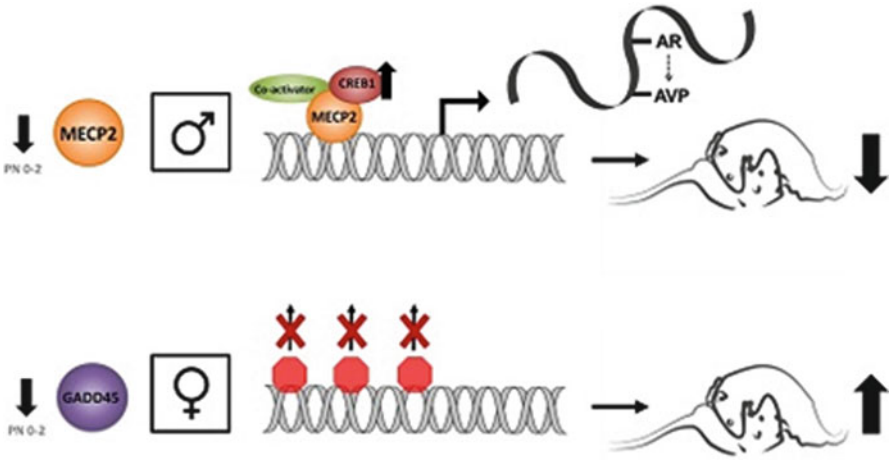

Fig. 9.6 *Neuroepigenetic regulation of sex differences in social play behavior.* Decreasing the DNA methyl-binding protein, MeCP2, decreases play behavior in juvenile males but not females. This may be mediated by MeCP2 association with CREB and a reduction in arginine vasopressin (AVP) and androgen receptor (AR) expression as opposed to the more traditional role for MeCP2 as a transcriptional repressor (based on Forbes-Lorman et al. 2012). Alternatively, decreasing the demethylating enzyme GADD45 dramatically increases play behavior by females but has no effect on males, suggesting higher DNA methylation represses play in females (based on Kigar et al. 2015). Drawing of play courtesy of Anthony Auger

et al. 2006; Kurian et al. 2010). The amount of maternal care differs for male and female rat pups, as mother rats preferentially lick and groom male offspring more than female offspring (Moore and Morelli 1979). Alteration of somatosensory stimulation associated with licking and grooming governs sex differences in ERα promoter methylation and subsequent expression in the developing amygdala (Edelmann and Auger 2011). The effect of stimulated maternal grooming in males and females on methylation of three CpG sites within exon 1b ERα promoter region was limited to sites near the consensus signal transducer and activator of transcription (STAT) 5 binding region which regulates ERα transcription. Enhancing female somatosensory stimulation associated with maternal grooming produced ERα methylation of these CpG sites that resembled patterns found in males. While gonadal hormones are responsible for modulating many sex differences in the amygdala and throughout the brain, these findings indicate the importance of environment in regulation of sexual differentiation and a role for epigenetic mechanisms in that regulation.

Thus, multiple convergent observations suggest epigenetic factors, particularly DNA methylation and subsequent protein recruitment, which contribute to sexual differentiation of the developing amygdala and social behavior. Sex differences exist in the expression of the methyltransferase DNMT3a, whereby males express significantly less of the protein at least partly as a result of hormone exposure. DNMT3a, being responsible for de novo methylation occurring during the critical

period of sexual differentiation, likely contributes to similar trends in reduced male expression of the methyl-binding protein MeCP2 and corepressor NCoR. Within the developing amygdala, MeCP2 and NCoR play an important, yet apparently opposing, role in the organization of juvenile social play behavior. In males, transient reductions in MeCP2 reduces juvenile social play behavior and also causes a temporary decrease in AR expression and a lasting reduction in AVP expression suggesting here MeCP2 acts to increase expression, potentially through interactions with cAMP pathways. Alternatively, transient disruption of NCoR expression produces increased juvenile social play behavior and therefore may be acting in a more traditional repressive role. Though many of these differences are a result of hormonal exposure during the critical period, early neonatal environment also contributes to programming of sex differences, as simulated maternal grooming masculinizes patterns of CpG site methylation in ERα. These diverse and sometimes opposing observations demonstrate the complexity and challenges of understanding how epigenetics regulates sex differentiation of social behavior.

9.5 Epigenetics of Sex Differences in the Hippocampus

In the preoptic area, the magnitude and impact of sex differences are unambiguous, and a great deal of the mechanism by which these occur is also being elucidated. But the hippocampus is far more complex in every domain. It has been long established that the hippocampus is a critical brain region for two distinct functions, spatial learning and stress responsiveness. Both of these end points differ on average to some degree in males and females, but both are also potently impacted by prior experience, current context, and circulating steroid levels at that time (Shors et al. 2001, 2004; Andreano and Cahill 2009). Thus, any sex difference that is observed in either the neuroanatomy or physiology of the hippocampus must be viewed through a multifaceted lens, and the same can be said to be true for any behavioral responses influenced by this brain region. The centrality of epigenetics to hippocampus-dependent memory formation has been firmly established, helping to explain how memories, whether good or bad, can endure for a lifetime (Zovkic et al. 2013). This is also true for estradiol-modulated hippocampus-dependent memory formation in the adult female (Fortress and Frick 2014). Developmentally, the hippocampus is subject to hormonal modulation in a manner that appears analogous to the canonical organizational/activational hypothesis that characterizes the sexual differentiation of the preoptic area and hypothalamus (Williams et al. 1990), but this analogy falls apart upon closer examination. The sexual differentiation of copulatory behavior is organized by higher gonadal steroids in the male preoptic area during the perinatal sensitive period and is activated by adult circulating gonadal steroids. But when gonadal steroids are measured in the developing hippocampus during this same period, there are no sex differences in the amount of testosterone, dihydrotestosterone, or estradiol, and the levels of the latter are exceedingly low,

10-fold less than that found in the preoptic area (Konkle and McCarthy 2011). But there is a profound sex difference in the rate of neurogenesis during the sensitive period, with more new neurons being born in the male hippocampus than the female, and if females are treated with exogenous estradiol, their rate of neurogenesis increases to that of males (Bowers et al. 2010). Treatment of males with the same dose of estradiol has no effect. Moreover, if either estradiol production or the estrogen receptor is antagonized in males, there is a profound reduction in neurogenesis, but there is no impact of the same treatment on females. So we have a conundrum; there is a sex difference in neurogenesis, neurogenesis is affected by estradiol, but there is no sex difference in estradiol. While there are many potential explanations for a sex difference in sensitivity to estradiol (i.e., number of receptors, co-activators, etc.), we postulate there is a differential epigenetic control of neurogenesis in males and females that involves repressing genes that promote proliferation in females and derepressing genes that promote both proliferation and neuronal differentiation in males. This duality of control could be mediated in part by microRNAs which regulate gene expression by binding to messenger RNA and either sterically blocking translation or promoting degradation of the mRNA (McNeill and Van Vactor 2012; Akerblom and Jakobsson 2013; Sun et al. 2013). Sex differences in microRNAs are found in adult brains (Morgan and Bale 2012). We examined a panel of hand-selected microRNAs implicated in cell proliferation and/or differentiation and found the majority of them were higher in the neonatal female hippocampus (McCarthy et al. 2015). Thus, males and females could have the same rates of transcription of estrogen-sensitive genes, but in females the production of proteins critical for proliferation and differentiation may be dampened by the higher levels of microRNAs. While an appealing explanation for a hormonally mediated sex difference in the absence of a sex difference in hormones, this only kicks the can a little further down the field as the next question is, what controls the sex difference in microRNA levels? Suggesting that estradiol controls microRNA level would seem to be a circular argument and so that leaves sex chromosome complement. Intriguingly, there are some microRNAs that escape X inactivation (Song et al. 2009), suggesting that sexual differentiation of the hippocampus could be mediated by a combination of genetics and hormones.

9.6 Epigenetics and Canalization of Brain Sex Differences

The brain utilizes the full range of epigenetic tools and thereby effectively integrates experience into the transcriptome. Even infrequent but highly salient events can have enduring consequences in the form of post-traumatic stress disorder, addiction, and risk of some neuropsychiatric disorders. Epigenetics provides the link between experience and brain plasticity so that past becomes prologue and future physiological or behavioral responses are in part directed by previous changes to the epigenome (Sweatt 2013).

Epigenetics is also a mechanism evoked to explain the process of canalization, a biological process intuitively illustrated in the iconic Waddington's epigenetic landscape in which a ball poised at the top of a hill can only roll down one of multiple canals and once in that canal the choice of additional routes down the hill becomes limited (Waddington 1959). Evolutionary biologists use canalization in the context of species robustness, a concept invoked to explain how plants and animals maintain a narrow range of within-species phenotype despite a constantly changing environment and minor genetic anomalies. Agents of canalization vary both in type and in response to the type of challenge. For instance, deficits in protein folding or cellular localization induced by minor mutations and amino acid substitutions are corrected by chaperone proteins, such as heat shock proteins (Ruden et al. 2003; Rohner et al. 2013). MicroRNAs are also proposed as agents of canalization by creating a nonlinear relationship between gene expression and translation when below a threshold of saturation and above which that relationship shifts to linear (Posadas and Carthew 2014).

Many of the neuroanatomical sex differences we and others have documented since the early 1970s fit the definition of being robust in that the magnitude of the differences are generally large, on the order of one to twofold, and the variance within each sex is relatively low, usually less than 10 % of the mean. Together, these observations suggest many neuroanatomical sex differences, and by this we mean cell number, dendritic length and branching, synapse frequency or density, astrocyte and microglia morphology, neurochemical phenotype, etc., are not continuously distributed. In other words, there is not a continuum along which males and females fall; rather, there is a bimodal distribution. This has led us to propose that many sex differences in the brain are canalized (McCarthy et al. 2015). We further propose that the process begins with the default canal being female and that high gonadal steroids in the male early in development divert him to a separate canal along which all other choices are made. Entrance into that canal involves at least in part a removal of repressive DNA methylation marks.

There are multiple and nonexclusive potential capacitors of canalization of brain sex differences. Hormones act via nuclear transcription factor receptors, and a hallmark of their activation is association with heat shock proteins, including hsp90 which may enable activity at the membrane (Setalo et al. 2002). Ours and others' observations of sex differences in microRNAs raise the specter of noncoding RNAs as agents of canalization in the brains of males and females. Epigenetic changes to the DNA are of course the ultimate director of canalization, and our finding that highly methylated CpG sites are twice as numerous in the female POA as the male lays the groundwork for a profound yet still poorly understood canalization of this brain region which boasts the most numerous and robust of neuroanatomical sex differences. Lastly, experience itself has the potential to further canalize sex differences by reinforcing or even rewarding sex-typical, and perhaps gender-typical, behaviors. Male and female rodent pups are treated differently by the dam (Moore and Morelli 1979; Moore 1984; Bowers et al. 2013), as are human babies and children responded to in a gender-biased manner by both their parents and strangers (Hines 2010).

9.7 Summary and Conclusions

The field of neuroepigenetics is in its earliest stages as we tackle the combined complexities of the nervous system and the multitude of modifiers of gene expression. The challenge is increased still further when the dynamics of development are introduced and an attempt made to understand how early epigenetic changes are established as well as how they may or may not be maintained. Nonetheless, some principles are already beginning to emerge. First is that masculinization of sexual behavior appears to involve an emancipation from repressive DNA methylation. This is evident in the ability of DNMT inhibition to masculinize copulatory behavior (Fig. 9.1). However, the relationship between DNA methylation, gene expression, and sexual differentiation is far from straightforward as there are many mysteries to be solved. Among these is explaining how very early hormone exposure can impact DNA methylation patterns that emerge far later, even well into adulthood. What type of programming occurs to mediate these delayed effects, and what is the functional role of these epigenetic changes?

Insights into the contribution of histone modifications are emerging, and the aromatase gene appears to be regulated by both chromatin and DNA epigenetic marks. The challenge will be to develop a histone code for the male and female brain, a code that is likely to vary by region and perhaps by cell type. This will be a daunting task but could be highly fruitful as suggested by the genome-wide analyses of just one mark, H3K4me3, in which females had almost twice as many sites with higher levels than males, and in both sexes 10–12 % was X-linked (Shen et al. 2014).

Both the genome-wide H3K4me3 analyses conducted by Shen et al. and the GWBS conducted by Nugent et al. generated so much data as to be overwhelming, with the time and resources required to mine it more deeply being the major limiting factor. But some would say the data are already flawed for two reasons. First is that in both cases the analyses are on a mix of cell types that includes many varieties of neurons, astrocytes, endothelial cells of the vasculature, and the innate immune cells of the brain, microglia. Having such a multitude of cell types could be just adding noise to a complex system, but they could also be creating misleading signals. In other words, an apparent epigenetic difference between samples may be a by-product of slight shifts in population cell type. This question also plagues other fields of epigenetics, and powerful new statistical tools are being developed to deconvolute complex data sets and control for variance in cell type. Initial approaches were limited by the requirement of a reference data set on purified cell preparations, but this would be exceedingly difficult in neuroscience as there is no “prototype” neuron. More recently, methods relying on orthogonal relationships and bootstrapping have successfully deconvoluted mixed cell data sets, and these approaches could prove invaluable to neuroepigenetics (Zou et al. 2014; Houseman et al. 2015). Second is that in each case the analyses are a snapshot in time of a highly dynamic system. We will never know if the comprehensive transcriptome described at time A is also true for time B, and the difference between time A and time B could be something as simple as morning versus evening, spring versus fall, and 2 days old versus

3 days old. However, every approach has its shortcomings, and so the challenge to neuroendocrinologists studying neuroepigenetics will be to remain both cognizant of those limitations and not intimidated by them. There are whole new vistas to be explored and now is not the time for reticence.

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Chapter 10
Differential Regulation of Androgen Receptor and DNA Methylation in Songbirds

Kazuhiro Wada

Abstract Animal behavior is a highly diverse phenotype constrained in species- or strain-specific manner. However, little is known regarding the molecular basis of evolution of animal behavior and its epigenetic contribution. Therefore, oscine songbirds possess a specialized neural system, called the song system for learning and producing species-specific vocal patterns. Approximately 3000 species of songbirds generate species-specific song patterns that have been learned from conspecific tutors. Song phenotypes are affected through domestication in some species, such as canary and the Bengalese finch. The Bengalese finch, a domesticated strain, produces a distinct song pattern with more complex syntax from the white-rumped munia, a wild-type strain. On the molecular level, the androgen receptor (*AR*) is differentially expressed in the basal ganglia vocal nucleus between these two strains and even within the domesticated population. Consistent with the difference of *AR* expression, an epigenetic modification, the state of DNA methylation differs in the region upstream of the *AR* gene in these populations. This differential *AR* expression level is correlated with a song phenotype: the mean coefficient variance of syllable-interval duration. The regulation of distribution of syllable-interval duration is recognized as one of the key phenotypic differences in the evolution of complex vocal syntax. The evidence provides insights into the molecular basis of behavioral evolution via the regulation of hormone-related genes and demonstrates a potential contribution of epigenetic modifications for behavioral phenotype regulation.

Keywords Domestication • Vocal evolution • Basal ganglia • Species difference • Song pattern

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10.1 Introduction

Although species-specific behavior with individual variations is always observed in animals, the precise neural mechanisms of this authenticity and variability are unknown (Katz and Harris-Warrick 1999). Characterizing the molecular basis for strain- and species-specific behavior, including epigenetic regulation for individual variety, is a crucial step to understand the evolution of animal behaviors.

Approximately 3000 species of songbirds generate species-specific song patterns that have been learned from conspecific tutors. Furthermore, domestication of the wild songbird affects song phenotypes. The Bengalese finch (*Lonchura striata* var. *domestica*, BF) is derived from the wild white-rumped munia (*L. striata*, WRM) and has been domesticated for more than 250 years in Japan. The effect of domestication and the selection for parenting behavior and white color morphs resulted in distinct differences in the song patterns of these two strains (Fig. 10.1a). WRMs sing stereotypical songs that have a fixed sequence with garbled song syllables. Conversely, despite some differences among individuals, the BF songs have a complex syntax with acoustically diverse chunks of syllables interspersed with diverse transitions (Okanoya 2004) (Fig. 10.1b).

Regardless of the diversity in song phenotypes, these songbirds employ similar specialized neuronal pathways for learning and expressing their songs, called the song system (Fig. 10.1c). In particular, the song system comprises the forebrain song nuclei that have similar topological, anatomical, functional, and connectivity characteristics in different songbirds. These nuclei are subdivided into two pathways. The posterior vocal pathway connects the forebrain to the brainstem vocal nuclei that is similar to the mammalian motor pathway (Nottebohm et al. 1976; Jarvis et al. 2005) (Fig. 10.1c). This pathway controls the motor neurons that produce sounds while modulating breathing. The anterior vocal pathway forms a pallial–basal ganglia–thalamic loop, which is similar to the mammalian cortical–basal ganglia–thalamocortical circuit and is necessary for song learning (Fig. 10.1c). The

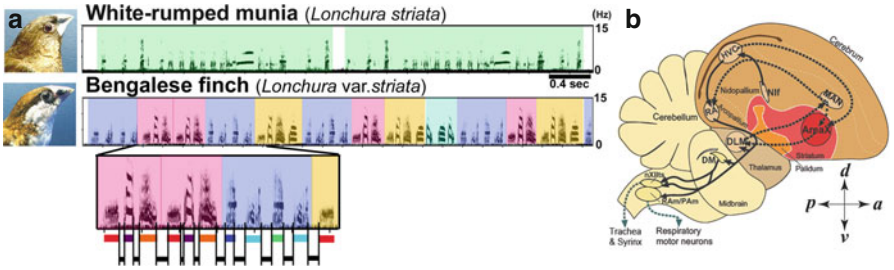

Fig. 10.1 Song patterns of white-rumped munia (WRM) and Bengalese finch (BF). (**a**) A typical example of song patterns of WRM and BF. The different colors in the BF song represent different types of syllable chunks. *Horizontal bars* of different colors represent individual syllables, and *black horizontal bars* represent inter-syllable durations. (**b**) Brain diagram of song pathways. *White solid lines* show the connections of the posterior vocal motor pathway, and *white dashed lines* show the anterior vocal pathway

anterior vocal pathway includes the basal ganglia nucleus Area X and is involved in real-time control of song production (Kobayashi 2001; Kao and Brainard 2006), modification (Kao et al. 2005), and learning (Bottjer et al. 1984; Scharff and Nottebohm 1991; Andalman and Fee 2009).

Focusing on the highly conserved neural circuits and distinct diverged vocal phenotypes between wild and domesticated strains in songbirds should enable the identification of the molecular basis responsible for the diversification of learned vocal phenotypes.

10.2 Androgens and Its Receptor in Songbirds

In songbirds, androgenic hormones expressed in the forebrain song nuclei possess functional links that affect singing behavior and forebrain song nuclei (Arnold 1975a, b; Nordeen et al. 1987; Gahr and Konishi 1988). The song system is highly sexually dimorphic (Nottebohm and Arnold 1976). Only males sing; and the song nuclei are much larger in male songbirds than in females in the temperate zone. In the zebra finch (ZF), removal of the testis affects the size of the song nuclei, and the amount of singing is reduced, although the birds still learn to imitate songs and to sing (Arnold 1975b). The tempo of the song is slowed by castration, and high testosterone concentrations reverse this effect (Arnold 1975a). Furthermore, chronic excessive testosterone during development impairs song learning, resulting in a decreased number of syllables (Korsia and Bottjer 1991). Although accumulated evidence suggests the association between androgens and song phenotypes, their causal relationships are still unknown.

Androgen receptor (*AR*) is a member of the steroid receptor superfamily of ligand-dependent transcription factors that bind cognate DNA sequences called androgen-responsive elements. The transcriptional effect of *AR* prevails at hundreds of gene loci in the proximity of androgen-responsive elements (Kennedy et al. 2010). The level of *AR* expression is differentially regulated by testosterone in species- and brain region-specific manners in mammals and birds, including songbirds (Gahr and Metzdorf 1997; Fraley et al. 2010; Imamura 2011).

10.3 Differential *AR* Expression Between Wild and Domesticated Songbird Strains

A difference in *AR* expression was found in the song system between WRM wild strain and the BF domesticated strain (Wada et al. 2013). As in other songbird species, *AR* is expressed at very similar levels and patterns throughout the vocal nuclei in the posterior and anterior motor vocal pathways (such as HVC, MAN, and RA; see Figs. 10.1b and 10.2a) in both strains. However, *AR* mRNA expression markedly differs in the striatum vocal nucleus Area X (Fig. 10.2a), with very little

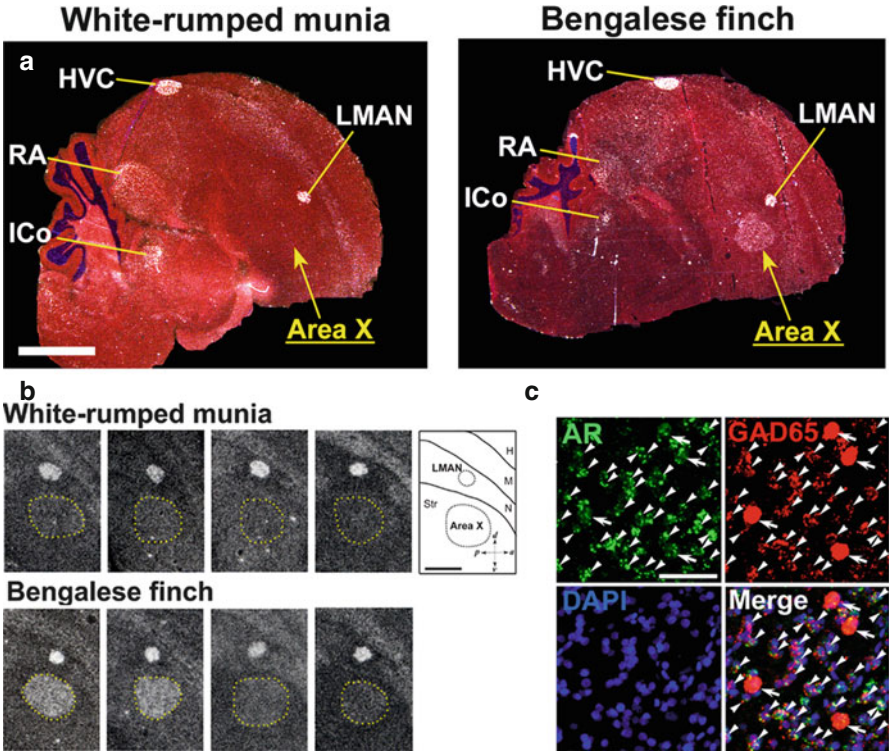

Fig. 10.2 Differential *AR* expression in WRM and BF. (**a**) Dark-field sagittal brain images of *AR* mRNA expression in WRM and BF. *White* color represents mRNA expression (scale bar = 2 mm). The striatum vocal nucleus Area X shows different expression levels between the two strains. (**b**) Inter- and intra-strain differences in *AR* expression in Area X. Magnified sagittal images of LMAN and Area X in WRM and BF adult males. Area X is highlighted with *yellow dotted lines* (scale bar = 500 μm). (**c**) *AR* is co-expressed with glutamic acid decarboxylase (GAD) 65, a GABAergic neuron marker, in Area X (scale bar = 40 μm)

expression in Area X of all WRMs and gradual differences in *AR* expression among BFs (Fig. 10.2b). These differences are Area X specific and are not observed in other song nuclei or in areas surrounding other vocal nuclei that are not directly involved in learned vocal production. This was the first example of a distinct difference of gene expression between wild and domesticated songbird species.

Two types of the GABA-positive cells are localized in Area X of songbirds, the small striatal spiny neurons and the large pallidal-like aspiny fast-firing neurons (Luo and Perkel 1999; Doupe et al. 2005). Both types of neurons can be labeled by the GABAergic inhibitory neuronal marker, glutamic acid decarboxylase (GAD) 65. Double-labeling in situ hybridization of *AR* and GAD65 showed that most *AR* (+) cells in Area X co-localized with GAD65 (+) neurons (Fig. 10.2c), indicating that *AR* expression is selectively and differentially regulated in GABAergic inhibitory neurons in Area X (Wada et al. 2013).

This result implies that the differential expression of the transcription factor *AR* may reflect functional differences in GABAergic neurons in Area X by regulation of its downstream genes within and between the strains. Currently, the *AR* target genes regulating the electrophysiological properties of these neurons remain to be identified. In mice, androgen- and *AR*-mediated neural signaling are critically important for modulating neural action potential frequency, spontaneous inhibitory postsynaptic current amplitude, and frequency with changes in GABAergic signaling in the medial preoptic area (Penatti et al. 2009), suggesting the potential of *AR* and androgens for modulating synaptic transmission in neuronal cells at specific brain sites.

10.4 Differential DNA Methylation Between Wild and Domestic Strains

To uncover the potential molecular mechanism that regulates differential *AR* expression in the striatum vocal nucleus Area X, we compared genome sequences and epigenetic states.

First, while increased testosterone levels increase *AR* expression in a motor circuit nucleus HVC in the white-crowned sparrow (*Zonotrichia leucophrys*) (Fraley et al. 2010), circulating testosterone concentrations were not significantly different in the two wild and domesticated songbird strains studied. Therefore, genomic variations in the regulatory region upstream of the *AR* gene were then considered as a possibility to associate with the transcriptional variation in *AR* levels. However, phylogenetic analysis of approximately 2.1 kbp upstream of *AR* did not reveal any strain-specific or *AR* expression-related difference. In addition, among the 71 identified single nucleotide polymorphisms (SNPs), no strain-specific SNPs were observed (Wada et al. 2013). Intriguingly, the region upstream of *AR* contained a typical CpG island (over 300 bp length including more than 50 % of G/Cs in total bases) at the putative 5′ UTR, with the CpG island shore located in the putative transcription regulatory region. DNA methylation states were then examined at the shore region and at the edge of the CpG island near the putative transcription start site of *AR*, using microdissected Area X of WRM and BF (a high- and low-*AR* expressing BF group). In the shore region of the CpG island (Fragment I in Fig. 10.3), less CpG methylation was found in high-*AR* expressing BFs than in low-AR expressing BFs and WRMs (Wada et al. 2013). Although it is yet unclear when the differences in DNA methylation states appeared, DNA methylation state indicates an epigenetic difference corresponding to hormone-related gene expression in a specific brain region associated with vocal learning. DNA methylation near the transcription start site of genes is generally involved in transcriptional silencing (Suzuki and Bird 2008).

The causal relationship between these methylation states and the regulation of *AR* expression remains to be further clarified in songbirds, after careful consideration of cell type specificity in Area X.

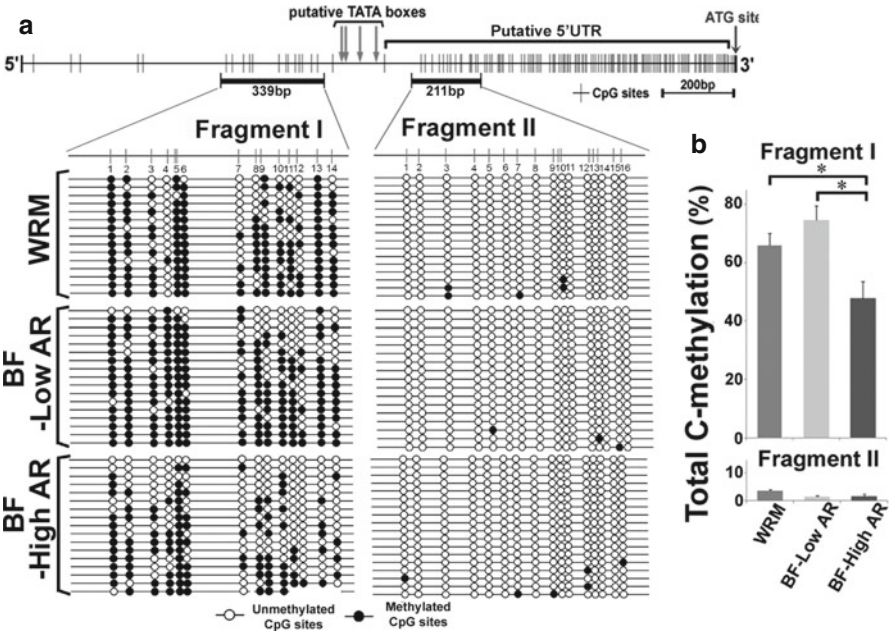

Fig. 10.3 Differential DNA methylation in the region upstream of *AR* between two strains. (**a**) Examples of DNA methylation states in two regions upstream of the *AR* gene. Fragment I is located at the CpG island shore. Fragment II is located at the putative 5′ UTR at the edge of the CpG site. *White* and *black* circles represent unmethylated and methylated CpG sites, respectively. (**b**) Frequency of methylated cytosine residues at all CpG sites in Fragments I and II of WRM, BF-low *AR*, and BF-high *AR*

10.5 Correlation of *AR* Expression in the Striatum Vocal Nuclei and Song Features

By examining the differences between the BF-low *AR* and BF-high *AR* groups in the song features including song linearity, song consistency, and syllable duration, the two groups showed a significant difference in the mean coefficient of variation (CV) of inter-syllable duration. Further, the correlation analysis between *AR* expression in Area X and the mean CV of inter-syllable duration for the two strains revealed a significant and positive correlation (Wada et al. 2013).

Further experiments are required to examine whether there is a direct relationship between *AR* expression in Area X and song features. *AR* in Area X may influence the variability in inter-syllable duration. This idea is consistent with a report that the basal ganglia–thalamocortical circuits, including Area X, are required for the generation of the random mode producing broadly distributed durations of syllables and inter-syllables (Aronov et al. 2011). Silent intervals in behavioral actions, such as inter-syllable duration, are considered to function as prosodic cues for segmentation and chunking when learning longer and more complex sequences (Williams and Staples 1992; Saffran 2002).

10.6 Implication of Neural Epigenetic Variation as a Driving Evolutionary Force of Animal Behavior

It has not been examined whether differential DNA methylation and *AR* expression were transgenerationally inherited in BFs. However, the differential *AR* expression in Area X of BFs was observed before the initiation of singing. Other possibilities include social interactions between siblings and parents in the nest, hatching dates, and/or the existence of an inherited or in ovo mechanisms. Further studies are required to elucidate the causal factors that regulate individual differential *AR* expression. While our knowledge on inherited epigenetic profiles is still limited in songbirds, stable inheritance of epigenetic variants has been demonstrated in rodents, humans, and birds (Franklin and Mansuy 2010; Franklin et al. 2010; Natt et al. 2012). Epigenetic variation has recently been considered a driving evolutionary force (Feinberg and Irizarry 2010). Variably methylated regions (VMRs) are present across species, individuals, and tissues (Irizarry et al. 2009). In addition, VMRs are rich in development-related genes and have been found to be related to phenotype, at least as far as the proximal gene (within approximately 500 bases from the start codon) (Feinberg and Irizarry 2010). The variability observed in the methylation state of the region upstream of the *AR* gene indicates characteristics of VMRs (Fig. 10.3). Furthermore, the findings suggest that VMRs may affect phenotypes not only at the tissue level but also at the specific cell type level.

Similar differences in *AR* expression in Area X have been observed in populations of ZF (Gahr 2004) and other songbirds, such as the spice finch (*Lonchura punctulata*), chipping sparrow (*Spizella passerina*), and ovenbird (*Seiurus aurocapillus*) (Fig. 10.4). Therefore, it appears that differential *AR* expression in the basal ganglia nucleus is not restricted to BFs and WRMs. Many species potentially have similar variations in *AR* expression among individuals, with differences in the distribution bias of *AR* expression varying in a species-specific manner. In specific

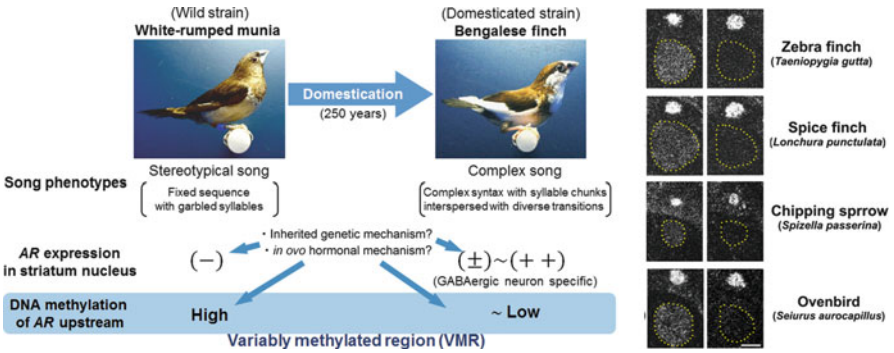

Fig. 10.4 Differential regulation of song phenotypes, *AR* expression, and DNA methylation between wild and domesticated strains. (**a**) Relationships between domestication, *AR* expression, and DNA methylation. (**b**) Similar differences in *AR* expression in Area X of other songbird species

neuron types, the distribution of key functional gene expression levels may have drifted between individuals and/or dispersed to other strains by epigenetic regulation of VMRs under specific environmental conditions, such as domestication. Epigenetic differences mediated by such important changes in gene expression could be a powerful catalyst for the evolution of behavior in phylogenetically constrained nervous systems.

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Chapter 11
Epigenetic Regulation of the *GnRH* and *Kiss1* Genes

Joseph R. Kurian

Abstract This chapter focuses on epigenetic regulation of the *GnRH* and *Kiss1* genes in the context of neuronal development, puberty onset, and reproductive function. Diverse epigenetic phenomena including the formation of chromatin loops, activation of bivalent domains, maintenance of "stable" histone modifications, and DNA methylation, active demethylation, and hydroxymethylation dynamics are covered. When possible, particular effort is made to focus on how these phenomena may be related to each other and which enzymes or binding factors are involved in these processes. In highlighting similar epigenetic mechanisms between these distinct peptidergic neuronal populations, we hope to impress upon the reader the potential relationships between those mechanisms and the development of hypothalamic peptidergic neurons in general. While this chapter does not cover relationships between peptidergic systems and psychiatric conditions, it does cover epigenetic mechanisms related to puberty. Because puberty represents a developmental period when neuropsychiatric diseases commonly present, understanding the epigenetic processes operating in the hypothalamus during that window could be instrumental to deciphering the onset, exacerbation, or resolution of neuropsychiatric diseases.

Keywords Gonadotropin-releasing hormone (GnRH) • Kisspeptin • Puberty • Bivalent chromatin domain • DNA hydroxymethylation

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11.1 GnRH Neurons

Gonadotropin-releasing hormone (GnRH) neurons are critical relays in the axis controlling reproductive function. These neurons reside in the preoptic area and basal hypothalamus and project to the pituitary, where they release the decapeptide GnRH into the portal circulation. GnRH subsequently stimulates anterior pituitary release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which promote gametogenesis and gonadal steroidogenesis in both sexes as well as ovulation in females. In order to properly stimulate gonadotropin release, GnRH must be supplied to the portal circulation in sufficient, episodic pulses. In fact, the onset of puberty and maintenance of reproductive function absolutely depend on the elevated and finely coordinated release of GnRH. While upstream mechanisms fine-tune GnRH neuronal activity, GnRH neurons also have an intrinsic capacity to release GnRH peptide in a pulsatile manner.

GnRH neurons reside in a complex milieu of other neurons and glia; this presents a challenge for studying the cellular and molecular mechanisms of neuronal differentiation and function. Fortunately, the unique ontogeny of GnRH neurons provides an opportunity to isolate a GnRH neuronal population for in vitro studies. In the rhesus monkey, these neurons differentiate from progenitor cells in the nasal placode between embryonic (E) days 32–34 (Ronnekleiv and Resko 1990; Quanbeck et al. 1997). GnRH neurons subsequently begin migrating into the brain at about E36 and settle down in the hypothalamus by E55 (Terasawa et al. 2001). Isolating the nasal placode after E34 but prior to migration provides a neuronal population that consists of entirely GnRH neurons. We found that placode tissue isolated on E36 developed typical patterns of mature activity (e.g., GnRH peptide release; Terasawa et al. 1993, 1999; Kurian et al. 2010a) after about 2 weeks in vitro. Wray and colleagues, who developed a similar murine in vitro culture model (Fueshko and Wray 1994), have also reported a period of gradual maturation after isolation from the nasal placode (Constantin et al. 2009). In addition, they report that development of GnRH peptide release patterns is paralleled by increasing GnRH gene expression and peptide biosynthesis (Maurer and Wray 1997; Moore and Wray 2000). A question arises. What mechanism triggers increasing gene expression during GnRH neuronal development?

11.2 DNA Methylation in GnRH Neurons

The genetic control of GnRH gene expression is well characterized and depends on several cis sequences in the 5′ region of the gene. The rat gene contains a neuron-specific enhancer region between −1863 and −1571 (Kepa et al. 1992, 1996b; Clark and Mellon 1995; Whyte et al. 1995). This spans a major region of homology between the rat (−1786 to −1559) and human (−2766 to −2539) genes. Interestingly, this portion of the human gene does not appear to enhance gene expression; in fact,

based on serial truncations of the 5′ human GnRH gene in luciferase assay constructs, it appears that this region impedes enhanced gene expression (Kepa et al. 1996a). Importantly, this area has sequence similarity to a 5′ portion of the rhesus monkey GnRH gene. We noticed that this distal 5′ region of the rhesus monkey gene contains a 243-bp segment (−2126 to −1863) that has 60 % GC content and a CpG (cytosine-guanine) dinucleotide observed to expected ratio of 0.65 (14 CpG sites). These characteristics define the region as a CpG island (CGI; Gardiner-Garden and Frommer 1987). CGIs, when associated with gene promoters, are related to the epigenetic regulation (DNA methylation) of gene expression (Deaton and Bird 2011).

DNA methylation is the covalent addition of a methyl (−CH3) group to nucleotides. Mammalian DNA methyltransferase (DNMT) enzymes catalyze this reaction. Mammalian DNA methylation occurs primarily at the 5′ carbon of cytosines in CpG dinucleotides and to a lesser extent at CpH (H = A, T, C) dinucleotides (Ramsahoye et al. 2000; Guo et al. 2014). There are three primary DNMT enzymes (1, 3a, and 3b), each critical to development as demonstrated by embryonic or early postnatal lethality in monogenic null mouse models (Li et al. 1992; Okano et al. 1999). DNMT1 is responsible for faithful maintenance of DNA methylation after replication through cell division (Bestor and Ingram 1983; Bestor et al. 1988; Hermann et al. 2004). DNMTs 3a and 3b are de novo methyltransferases responsible for newly acquired methylation such as during the initial establishment of methylation patterns during early embryonic development (Okano et al. 1998, 1999) and throughout neuronal maturation and aging (Guo et al. 2014). Once established, DNA methylation can have several impacts on gene transcription. Methylated DNA can directly alter transcription factor recognition of cis sequences or attract methyl-binding proteins, thereby blocking genomic locations from transcription factor assembly. In addition, methyl-binding proteins (e.g., MeCP2, Mbd2, Kaiso) interact with histone-modifying factors to alter chromatin structure.

Until our recent studies (Kurian et al. 2010a), there were no reports of the epigenetic aspects of GnRH neuron maturation or function. The CGI in the 5′ region of the rhesus monkey *GnRH* gene was suggestive to us that DNA methylation had some role in neuronal function (Fig. 11.1). We hypothesized that increasing peptide release during in vitro maturation of GnRH neurons would be related to increased gene expression and changing DNA methylation patterns across the rhesus monkey *GnRH* gene, particularly within the 5′CGI. As suspected, we found that GnRH mRNA levels were low at day 0 but rose dramatically by day 20 of in vitro cultures. This increase was paralleled by a dramatic decrease in CpG methylation status in the 5′ CGI. We also found that a comparable phenomenon occurs across puberty. Similar to the previous observation by Plant and colleagues showing that GnRH mRNA levels increase between juvenile and pubertal stages in the medial basal hypothalamus (MBH) of orchidectomized male rhesus monkeys (El Majdoubi et al. 2000), we also observed that GnRH mRNA levels increase in the male MBH across puberty. Furthermore, methylation status of the 5′ CGI of the *GnRH* gene was lower in adult compared to prepubertal male rhesus monkeys (Kurian et al. 2011). This suggests to us that developmental rises in GnRH gene expression are at least partly the result of DNA demethylation across a CGI in the GnRH gene.

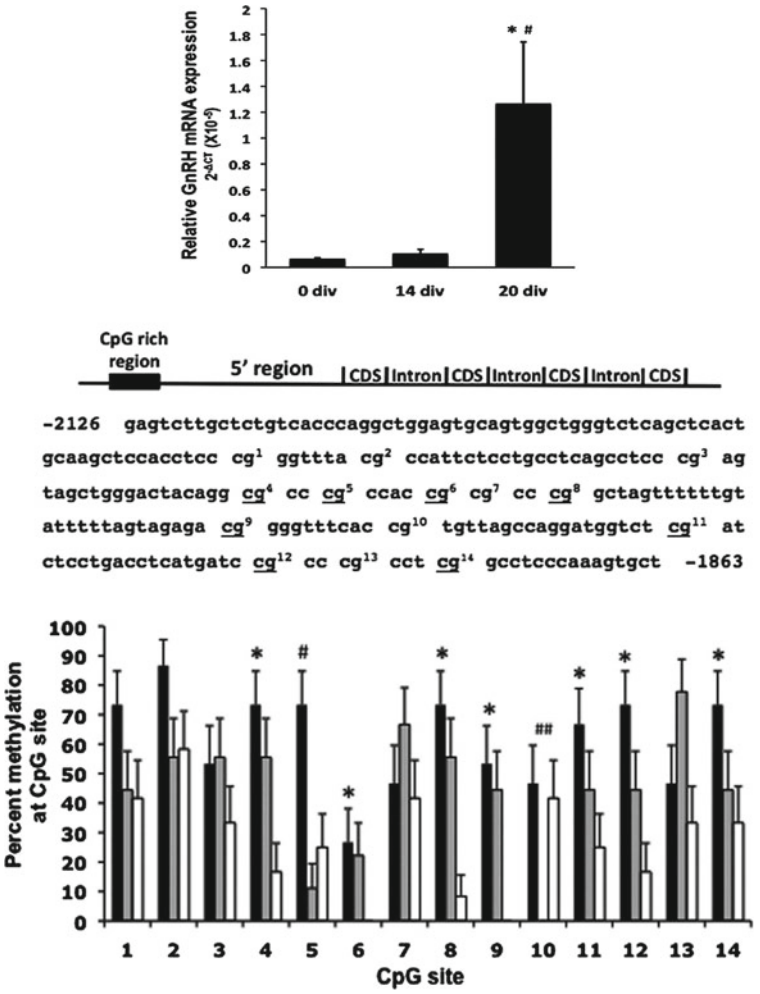

Fig. 11.1 Embryonic development of primate GnRH neurons is marked by a dramatic shift in *GnRH* gene expression and active demethylation of a 5′ CpG island (*Top portion*). Total RNA was extracted from in vitro nasal placode cultures (in vitro neuron development cultures) at 0, 14, and 20 div. GnRH mRNA levels, measured by quantitative PCR, started to increase after 14 div, reaching the highest level at 20 div. (*$P<0.05$ vs. 0 div; #$P=0.05$ vs. 14 div) (*Middle portion*). A Schematic representation of the rhesus monkey *GnRH* gene depicting the location of the 5′ CpG island (CGI) and nucleotide sequence of this region. This region is the only classifiable CGI within 2500 bases upstream of the *GnRH* gene transcription start site. CpG sites are indicated with numbers corresponding to the CpG sites in the bottom graph (*Bottom portion*). GnRH neurons dissected from the nasal placode region of two rhesus monkey embryos at E36 and E37 were plated and then harvested on 0, 14, or 20 div. DNA extracted from pooled samples (four cultures) at each time point was bisulfite sequenced. Percent changes in methylation at each CpG site on 0 div (*black bars*), 14 div (*gray bars*), and 20 div (*white bars*) are shown. CpG methylation status was significantly higher at 0 div compared with 20 div at sites 4, 5, 6, 8, 9, 11, 12, and 14 (*$P\leq0.01$). CpG methylation status was significantly higher at 0 div compared with 14 div at site 5 (#$P<0.05$) and at 0 div compared with 14 div but not 20 div at site 10 (##$P<0.05$)

When evaluating these findings, it is important to consider the distinct pattern of GnRH neuronal activity across development. In primates, GnRH release is elevated, as indicated by peripheral luteinizing hormone levels, during a brief perinatal period, but then decreases during juvenile development before gradually increasing again through puberty. Our findings suggest that each period of elevated GnRH release is related to demethylation of the *GnRH* gene CGI. Specifically, in vitro maturation is representative of embryonic development leading to elevated activity during the perinatal period, while measurements in MBH tissue are indicative of developmental changes across pubertal maturation. The decrease in methylation status across puberty is indicative of two potential scenarios. One possibility is that the process initiated in embryonic development might stall during the juvenile period and subsequently resume at puberty onset. Alternatively, the CGI may become re-methylated during juvenile development and again demethylated at puberty onset. The latter scenario would suggest that lower CpG methylation status must be maintained to enable elevated GnRH gene expression. Consequently, a mechanism responsible for DNA demethylation and perhaps another mechanism responsible for maintaining hypomethylated DNA might both be necessary for the transition to puberty and maintenance of reproductive function. A comparison of CpG methylation status between perinatal and early pubertal MBH tissue will be necessary to differentiate between these two scenarios. Nonetheless, our current findings suggest that DNA demethylation is an important aspect of GnRH neuronal development and function.

Given the postmitotic/nondividing state of GnRH neurons in our in vitro cultures and across the pubertal transition measured in MBH samples, the process of DNA demethylation we discovered must be active. The mechanisms responsible for active DNA demethylation are not well characterized, and until recently skepticism has remained over the existence of this process in mammalian systems. DNA demethylation is achieved via two general mechanisms, passive or active. Passive demethylation occurs through cell divisions and interruption of maintenance methyltransferase activity. Current pharmaceutical approaches for DNA demethylation target this mechanism. For example, 5-azacytidine (5-aza) is metabolized and subsequently incorporated into DNA, where it then acts as a substrate that covalently traps DNMTs after methyl transfer to the 5-aza nitrogen (Schermelleh et al. 2005; Svedruzic 2008). Active demethylation is not well characterized. Several mechanisms are proposed, and intense efforts to validate or better characterize these mechanisms continue. For thorough background, we suggest a recent review (Wu and Zhang 2010) that outlines several promising pathways discovered during the past decade. Presently, a well-accepted mechanism is the sequential enzymatic process beginning with oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which is carried out by any one of three ten-eleven-translocation enzymes (Tet 1–3). 5hmC is a stable epigenetic modification, though under certain circumstances it is recognized and excised by thymine DNA glycosylases. Base excision repair subsequently completes the transition. Importantly, brain tissue, and particularly the hypothalamus, has the highest reported 5-hydroxymethylcytosine (5hmC) tissue abundance (Branco et al. 2012).

Emerging physiological evidence also supports a role for Tet enzymes in neuroendocrine development and particularly the control of reproductive function. For example, Tet1 knockout mice exhibit deficits in fecundity. This effect is primarily a female-specific abnormality, as typical litter sizes result from crossing wild-type females with Tet1 knockout males, whereas wild-type males mated to Tet1 knockout females produce significantly fewer offspring per litter (Dawlaty et al. 2011). A subsequent report suggests this defect in fecundity might be the consequence of abnormal progression of female germ cell development through the second meiotic division prior to ovulation (Yamaguchi et al. 2012). Interestingly, stimulation of the second meiotic division and ovulation requires a GnRH-driven LH surge (Mehlmann 2005). Consequently, given the apparent active DNA demethylation associated with GnRH neuronal development, the effect of Tet depletion on abnormal reproductive function might be the consequence of altered Tet-mediated epigenetic differentiation in the neuroendocrine hypothalamus. While hypothalamic expression patterns of Tet enzymes are not yet reported, we recently found that basal hypothalamic Tet2 expression increases across puberty in female rhesus monkeys (Kurian and Terasawa; unpublished observation). That this deficit in fecundity was even more pronounced in double knockout (Tet1 and Tet2 knockout) mice (Dawlaty et al. 2013) gives further credence to the suggestion that Tet enzymes are active in the hypothalamus to promote neuronal maturation toward stage-specific reproductive function.

11.3 Histone Modifications in GnRH Neurons

Histones are an integral component of nucleosomes, the primary units for genome organization or compaction. Consequently, these proteins, through interaction with DNA, have a critical role in determining gene expression patterns. There are four primary classes of histones, 1 through 4. Histones 2 through 4 are components of the core octamer, which DNA circumnavigates in about 146 base pairs to form a single nucleosome. Histone 1 is a scaffold protein, which tightly packages nucleosomes when present. Several variants of this histone are distinguishable by sensitivity to hormones (Banks et al. 2001). Histone 2 also has several variants including A, AX, and B. Histone 2AX is particularly intriguing in the context of neuronal maturation (Lee et al. 2010) and function based on its association with activity-dependent DNA double-strand breaks in neurons (Suberbielle et al. 2013). Histones 3 and 4 complete the nucleosome octamer with histone 3 the most heavily investigated in the realm of neuroendocrine function. Histone 3 has two variants, H3.3A and H3.3B. These variants are integral to DNA replication-independent histone switching, which would be presumed an important mechanism in regulation of postmitotic cell activity. To date, to our knowledge, nothing is reported regarding the relationship between histone switching and hypothalamic neuronal maturation or function.

Histone proteins package DNA largely due to their predominant positive charge attracting negatively charged DNA. Posttranslational modifications (PTMs) alter

the strength of that attraction and recruit or repel transcriptional machinery and histone-modifying enzymes. Consequently, these PTMs alter gene accessibility and rates of transcription. Several known modifications include acetylation, phosphorylation, methylation, ubiquitination, SUMOylation, and GlcNAcylation. To date, measurements of histone PTMs in neuroendocrine systems have focused on acetylation and methylation. Acetylation leaves a more negative charge on histones and consequently promotes transcription. Methylation is neutral and, depending on the location and degree (mono-, di-, or trimethylation), can either promote or inhibit transcription.

Mellon and colleagues were the first to report a pattern of permissive histone modifications enabling elevated or mature GnRH gene transcription (Iyer et al. 2010). Their studies capitalized on the distinct stages of development between two GnRH neuronal cell lines. GN11 cells, originally isolated from a tumor in the mouse nasal placode (Radovick et al. 1991), express GnRH at very low levels, whereas GT1 cells, which were isolated from an analogous tumor in the mouse hypothalamus (Mellon et al. 1990), are characterized by mature activity patterns including elevated GnRH gene expression. In essence, comparisons between these two cell lines are similar to our evaluations of embryonic nasal placode-derived neurons from days 0 and 20 in vitro described above. They found that the GnRH gene promoter and enhancer regions in immature GN cells were more heavily associated with a repressive histone modification: histone 3 (H3) lysine 9 (K9) di-methylation (me2). On the other hand, the same genomic regions in GT1 cells were associated with the permissive H3K9 acetylation and H3K4me3 PTMs. For comparison, they also evaluated these histone PTM patterns in a non-neuronal (NIH3T3) cell line. The repressive PTMs were high in NIH3T3 cells, intermediate in GN cells, and low in GT1 cells. The presence of permissive PTMs was low and similar between NIH3T3 and GN cells but significantly higher in mature GT1 cells.

The intermediate repressive chromatin state in GN cells is indicative of a bivalent promoter, where both repressive and permissive histone PTMs maintain genes in a repressed albeit primed position. These chromatin domains were first described in embryonic stem (ES) cells and associated with developmentally regulated transcription factors (Azuara et al. 2006; Bernstein et al. 2006; Pan et al. 2007). Several recent reports, taken together, indicate that establishment of bivalent domains through polycomb repressive complex 2 (PRC2) and COMPASS families is critical for neural lineage differentiation from ES cells (Yu et al. 1995; Yagi et al. 1998; Glaser et al. 2006; Pasini et al. 2007; Shen et al. 2008). The PRC2 component, Ezh2, is responsible for H3K27 methylation, which is subsequently bound by the PRC1 complex to maintain trimethylation at H3K27. COMPASS complexes methylate H3K4. Together, these complexes are proposed to establish a bivalent promoter, with the heavily repressive H3K27me3 mark in close proximity to H3K4me3. Interestingly, this bivalent domain also appears related to the juvenile repression and subsequent activation of *Kiss1* (kisspeptin) gene expression during the pubertal transition in female rats (Lomniczi et al. 2013). As a major stimulant of GnRH release, this epigenetic regulation of *Kiss1* expression (discussed in detail below) may be instrumental in pubertal development. Together, this also suggests that bivalent domain activation may be a consistent mechanism across reproductive neuroendocrine systems.

11.4 Tet Enzyme Activity in GnRH Neurons and Reproductive Function

A recent discovery points to Tet enzymes as critical mediators of activation at bivalent promoters during neuronal differentiation. Specifically, while Ezh2 (the H3K27 methyltransferase component of PRC2) is critical for progression of neuronal precursor cells toward a neuronal fate, Tet2 or Tet3 appear to complete the process of differentiation (Hahn et al. 2013). In fact, these studies also found that accumulation of intragenic 5-hydroxymethylcytosine is associated with loss of H3K27me3 near regions with the most significant gene activation during neuronal differentiation. In addition, Tet2 was recently reported to promote H3K4me3 through association with the Set1/COMPASS complex (Deplus et al. 2013). Based on this background, our recent studies have focused on Tet enzyme activity in the context of GnRH neuron development and function. Initially we utilized the developmentally distinct GnRH neuronal cell lines. We discovered that Tet2 expression is low in immature GN11 cells, but substantially higher in mature GT1-7 cells. Subsequently, we found that overexpression of Tet2 in immature cells led to significant elevation of *GnRH* gene expression. This increase was potentiated when cultured cells were supplemented with glutamine, which enhances Tet2 activity by increasing intracellular concentrations of the cofactor alpha-ketoglutarate (Yang et al. 2014; Carey et al. 2015). Using chromatin immunoprecipitation, we evaluated Tet2 binding as well as H3K4me3 and H3K27me3 abundance across the mouse *GnRH* gene 5′ region (Kurian 2015; Fig. 11.2). Chromatin conditions were evaluated in four conditions: (1) GN11, (2) GT1-7, (3) GN11 cells overexpressing Tet2, and 4) GT1-7 cells with Tet2 eliminated by CRISPR-/cas9-mediated gene disruption. Tet2 binding was low in GN11 cells across the entire 5′ region, but significantly elevated in GT1-7 cells at the promoter- and neuron-specific enhancer region. The activating H3K4me3 modification largely mirrored Tet2 binding patterns, with significantly higher abundance in GT1-7 compared to GN11 cells. Overexpression of Tet2 in GN11 cells significantly increased Tet2 binding of the promoter and to a lesser extent the neuron-specific enhancer; H3K4me3 levels were also significantly higher at the promoter compared to non-transfected GN11 cells. Disruption of Tet2 in GT1-7 cells decreased Tet2 binding across the entire region; remarkably, H3K4me3 levels significantly dropped at the neuron-specific enhancer after Tet2 disruption in GT1-7 cells. H3K27 levels were largely consistent between all conditions at each site evaluated. All together, these results show that Tet2 influences bivalent promoter-specific histone modification patterns near the *GnRH* gene and contributes to the elevation and maintenance of elevated GnRH gene transcription (Fig. 11.3). Our subsequent studies evaluated the impact of Tet2 activity on puberty progression and reproductive function.

Using cre-lox technology, we recently generated mice with GnRH neuron-specific deletion of *Tet2* (gTKO animals). We expected that this would disrupt GnRH neuron epigenetic maturation and consequently the acquisition of reproductive competence (i.e., puberty onset and progression). Surprisingly, we found no evidence of pubertal disruption, though, remarkably, gTKO males exhibited an age-dependent decrease of

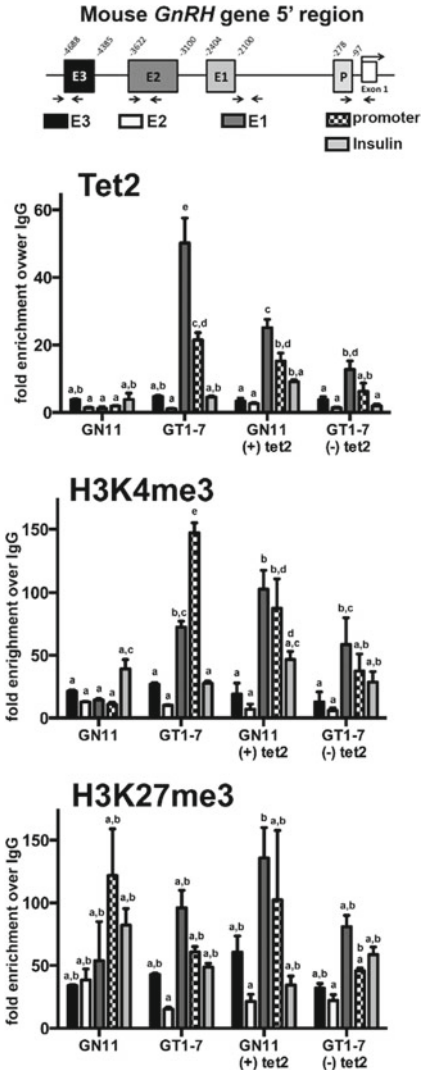

Fig. 11.2 Tet2 binds the mouse *GnRH* gene and promotes accumulation and maintenance of an activating histone modification (*Top portion*). Schematic diagram of the mouse GnRH gene showing relative locations of 3 enhancer regions (E1-3) and the gene promoter (P) [adapted from Iyer et al. 2010) (*Bottom graphs*). Graphs represent relative abundance of Tet2, H3K4me3, and H3K27me3 detected at each *GnRH* gene region using chromatin immunoprecipitation from immature GnRH neurons (GN11), differentiated GnRH neurons (GT1-7), GN11 cells transfected with Tet2 (GN11 (+) tet2), and GT1-7 cells with Tet2 knockdown (GT1-7 (−) tet2). Tet2 binds the promoter- and neuron-specific enhancer (E1) in GT1-7 and GN11 (+) tet2 cells. H3K4me3 is significantly more abundant at the promoter and E1 region in GT1-7 and GN11 (+) tet2 compared to GN11 cells. H3K27me3 abundance was similar across all cell lines at all regions evaluated. Comparisons were made within each chromatin-associating factor. Levels assigned with different letters are significantly different by Tukey post hoc after two-way ANOVA across all cell lines and genomic regions

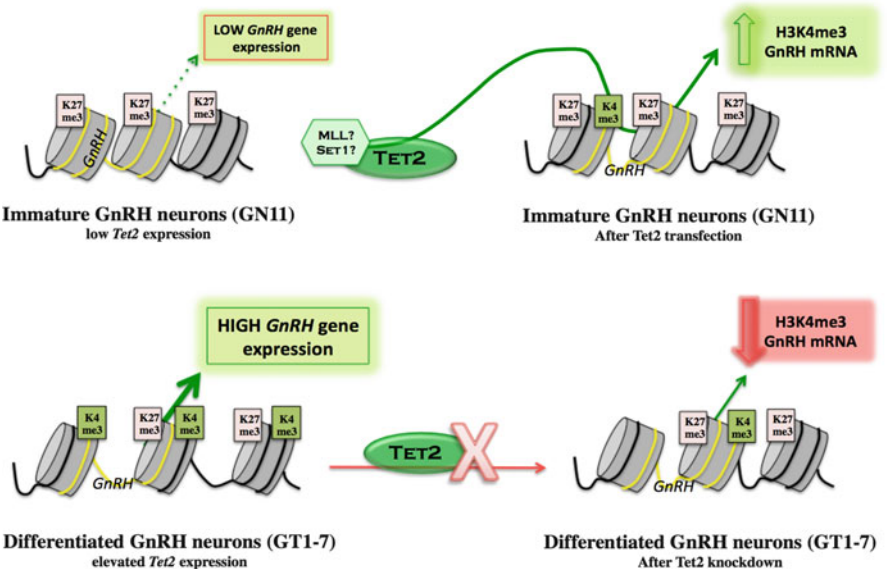

Fig. 11.3 Tet2 influences accumulation and maintenance of the activating H3K4 trimethylation in immature and differentiated GnRH neurons, respectively (*Top portion*). Immature GnRH neuronal cell lines (GN11) express *GnRH* at very low levels and less *Tet2* than differentiated GnRH neuronal cell lines (GT1-7). Increasing Tet2 expression in GN11 cells by transient transfection leads to an elevation of GnRH mRNA and H3K4me3 at the *GnRH* gene neuron-specific enhancer and promoter (*Bottom portion*). Knockdown of Tet2 expression in differentiated GnRH neuronal cultures (GT1-7 cells) leads to a loss of Tet2 binding near the *GnRH* gene promoter, loss of H3K4me3 abundance in the same region, and reduction in GnRH mRNA levels

plasma LH and impairment in fecundity that was not seen in wild-type animals (Kurian et al. 2014). Considered together with the loss of H3K4me3 abundance at the *GnRH* promoter after Tet2 disruption in the mature GnRH cell line, these physiological findings hint toward a novel concept. It appears that epigenetic patterns must be actively maintained, as opposed to simply a developmental process.

11.5 Kisspeptin Neurons

Kisspeptin peptides were originally coined "metastins" after they were discovered in the mid-1990s as novel ligands that suppressed breast and melanoma metastasis (Lee et al. 1996). Since that discovery, the biological roles of kisspeptin have broadened, most notably for their participation in reproductive maturation (puberty) and ovulation. In 2003, two independent reports revealed a novel signaling mechanism crucial to typical reproductive function. Specifically, mutations in the human gene encoding G-protein coupled receptor 54 (*GPR54* or *KISS1R*) were linked to the absence of puberty or severe disruption of pubertal progression (Seminara et al. 2003; de Roux et al. 2003). More recently, a familial mutation in *KISS1* (the gene

encoding kisspeptins) was linked to the impairment of pubertal progress (Topaloglu et al. 2012). Mice lacking either *Gpr54* or *Kiss1* exhibit delayed pubertal maturation and hypogonadotropic hypogonadism (Funes et al. 2003; d'Anglemont et al. 2007; Laptatto et al. 2007), whereas prepubertal exogenous kisspeptin exposures potently stimulate gonadotropin release in primates, rodents, and sheep (Guerriero et al. 2012; Dhillo et al. 2007; Gottsch et al. 2004; Messager et al. 2005; Navarro et al. 2005a, b; Shahab et al. 2005). These kisspeptin stimulations of gonadotropin secretion occur through activation of Gpr54 on GnRH neurons (Hrabovszky et al. 2010; Kirilov et al. 2013; Yeo et al. 2014), suggesting GnRH neurons are capable of elevated adult activity prior to pubertal onset and that developmental activation of kisspeptin systems may play more heavily into the timing of puberty. Consequently, based on the proposed environmental influence over pubertal timing, significant efforts are currently directed toward characterizing the epigenetic development and regulation of kisspeptin cells.

Kisspeptin cell bodies are concentrated in the rodent medial amygdala (Kim et al. 2011) and, to a much larger extent, the hypothalamic anteroventral periventricular nucleus and neighboring periventricular nucleus (AVPV) and the arcuate nucleus (ARC) (Gottsch et al. 2004; Kauffman et al. 2007; Smith et al. 2005b). Species differences exist, though nonhuman primate and sheep *Kiss1* gene expression and kisspeptin immunoreactivity exhibit distributions similar to rodents, with expression concentrated in the preoptic area (POA) and the ARC (Alçin et al. 2013; Ramaswamy et al. 2008; Shahab et al. 2005; Smith 2008; Smith et al. 2010). In humans, *KISS1* expression is mainly concentrated in a region homologous to the ARC as well as the medial preoptic area (Rometo et al. 2007).

Kisspeptin neuronal systems develop in regional and sex-specific programs. Specifically, whereas *Kiss1* is detectable in the rodent ARC by embryonic day 14 (Desroziers et al. 2012) and persists stably throughout postnatal and pubertal development (Poling and Kauffman 2013; Semaan and Kauffman 2015), AVPV *Kiss1* expression becomes apparent at postnatal day (PND) 10 (Semaan et al. 2010) with only a small amount of kisspeptin peptide detectable by PND 15 (Clarkson et al. 2009) but then substantially elevates prior to puberty onset (Semaan and Kauffman 2010, 2015). In addition, the appearance of *Kiss1* AVPV briefly lags behind in males and is significantly lower in pubertal and adult males compared to females (Kauffman et al. 2007; Semaan et al. 2010; Clarkson and Herbison 2006; Homma et al. 2009). The sex- and region-specific differences in kisspeptin expression are critical to sex-specific reproductive function and have therefore been a focus of early investigations of kisspeptin neuron epigenetic regulation.

11.6 Epigenetic Aspects of Sexual Differentiation of Kisspeptin Neurons

Sex differences in the brain are largely created during a perinatal "critical period" by a sex-specific steroid environment (Phoenix et al. 1959). During this critical period, a male-specific acute surge of testicular testosterone secretion coordinates

the masculinization/defeminization of the brain and associated physiology and behaviors (Simerly 1998, 2002). Importantly, prior to acute sex steroid exposure, the brain exhibits bipotential. As evidence, perinatal treatment of females can direct male-like brain development, whereas newborn male castration leads to a female-like brain in adulthood. The critical period extends through approximately postnatal day 10 in rodents, after which sex steroid manipulations no longer alter sexually dimorphic brain structures, though some brain traits appear to be influenced by steroid exposures during the pubertal stage (Schulz and Sisk 2006; Schulz et al. 2009).

Importantly, in addition to sex steroids, sexually dimorphic experiences during the perinatal critical period also seem to, at least, masculinize the brain. For example, male rodents experience a higher degree of maternal grooming than female littermates during this period. Subjecting female rats to a sex steroid surge or simulated maternal grooming in the first week of life is reported to masculinize the estrogen receptor-α (ERα) gene CpG methylation pattern and gene expression profile in the medial basal hypothalamus (Kurian et al. 2010b). That study was the first to report an equivalent impact of steroids and environmental influence over sexual differentiation of epigenetic patterns in the brain. This suggested that epigenetic mechanisms likely had a significant role in sexual differentiation of the brain in general. In fact, several "critical period" sex steroid exposures were soon after related to epigenetic factors including differentiation of BNST size and vasopressin fiber projections (Murray et al. 2011), male sexual behavior in rats and olfactory behavior in mice (Matsuda et al 2011), and steroid receptor and DNA methyltransferase 3a expression (Schwarz et al. 2010; Kolodkin and Auger 2011).

To date, only one study has investigated whether sexual differentiation of kisspeptin systems is driven by epigenetic factors. Specifically, Semaan, Kauffman, and colleagues evaluated whether histone deacetylation and DNA methylation contributed to sexual differentiation of AVPV *Kiss1* gene expression and cell number (Semaan et al. 2012). They pharmacologically blocked histone deacetylation during the postnatal critical period with valproic acid and evaluated male and female AVPV *Kiss1* expression in adulthood. While they observed the expected disruption of BNST differentiation (Murray et al 2011), and *Kiss1* gene expression was elevated in both sexes compared to same-sex controls, there was no effect on sexual differentiation per se. Whether the elevation of *Kiss1* expression in males had a functional impact (i.e., feminization of estradiol-induced LH surge potential) was not evaluated and remains an important question.

Semaan and colleagues (2012) also evaluated DNA methylation over 70 unique sites of the *Kiss1* gene in microdissected AVPV tissue. CpG methylation was significantly different, primarily in the putative *Kiss1* promoter region, and, remarkably, higher in females at every site with differential methylation. This finding might seem incongruent with the typical perspective of higher promoter methylation related to lower gene expression given that *Kiss1* expression is profoundly higher in females compared to males in this region. However, when considering the apparent importance of chromatin looping to AVPV *Kiss1* gene expression (discussed in following section), this sex difference in CpG methylation may play a critical role in

sexual differentiation of the kisspeptin system and female reproductive function in general. Importantly, these evaluations of CpG methylation did not consider the impact of perinatal hormone exposures, consequently, whether the perinatal hormone surge drives epigenetic differentiation of the *Kiss1* gene in the AVPV remains an open critical question.

11.7 Epigenetic Aspects of Estrogen Signaling on *Kiss1* Gene Expression

An essential aspect of female reproductive function is the ability to generate estradiol-induced LH surges. LH surges are critical to gamete maturation and ovulation and require intact estrogen receptor-α (ERα) signaling in AVPV kisspeptin neurons (d'Angelmont and Colledge 2010; Dubois et al. 2015). Remarkably, acute sex steroid exposures during adulthood have opposite effects on *Kiss1* gene expression in the AVPV and ARC. Whereas *Kiss1* expression is reduced in the ARC, it is significantly elevated in the AVPV after estradiol (E2) exposure in females (Smith et al. 2005a). This phenomenon was recently utilized to interrogate the epigenetic and genetic aspects related to E2-driven effects on *Kiss1* gene expression (Fig. 11.4).

First, using only adult female animals, Tomikawa and colleagues reported that E2 exposure increased ERα presence near the *Kiss1* promoter in the AVPV, but not in the ARC (Tomikawa et al. 2012). In addition, total histone 3 (H3) acetylation near the *Kiss1* promoter increased in the AVPV and simultaneously dropped in the ARC during E2 exposure. Interestingly, H3 acetylation in only the ARC also changed in a 5′ region upstream of the *Kiss1* promoter (increased acetylation) and a 3′ intergenic region (decreased acetylation) located downstream of the last exon of the *Kiss1* gene. These latter differences in H3 acetylation are probably the consequence of brain region-specific chromatin loop conformations. Specifically, using chromatin conformation capture assays, Tomikawa and colleagues exquisitely found that in the ARC, the *Kiss1* gene is held in a tight loop, with interactions between a 5′ region and a proximal 3′ region centered at the promoter. In contrast, no chromatin loop is apparent in the AVPV; however, during E2 exposure, a loop forms between the promoter and a distal intergenic 3′ region, later shown to be an enhancer in the AVPV. That same distal 3′ region also associates with the promoter in the ARC during E2; interestingly though, whereas that region is necessary for enhancing E2-driven AVPV *Kiss1* expression, it is also necessary for E2 inhibition of ARC *Kiss1* expression. We consequently refer to this region as a "transcriptional modifying region." Importantly, while histone acetylation is typically associated with increased gene expression, the ARC 5′ region exhibits increased H3 acetylation abundance even while *Kiss1* expression significantly drops. Examination of the DNA loop structures generated by E2 exposure in the ARC (Fig. 11.4) sheds light on this apparent dis-concordance. It is possible that the tight looping of DNA results in histone displacement without any loss of histone acetylation, thereby shifting the regional associations between the *Kiss1* gene and histone core. In essence, this

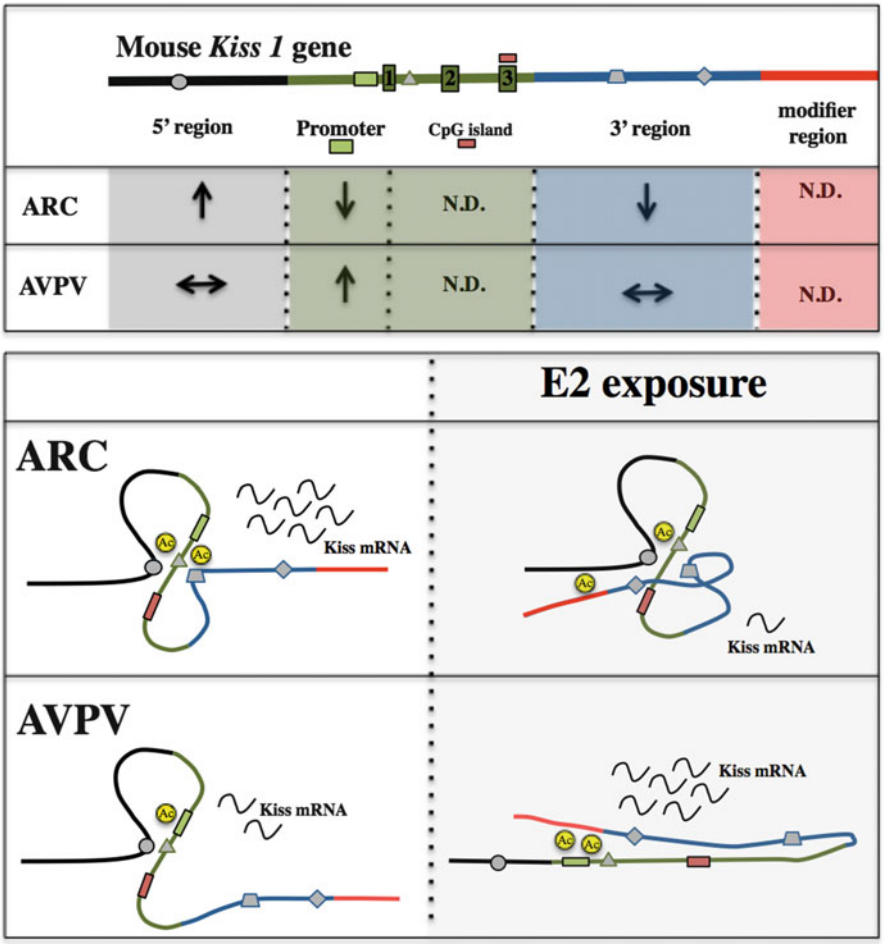

Fig. 11.4 Estradiol (E2) exposure has brain region-specific influence over *Kiss1* gene expression, chromatin conformation, and histone 3 (H3) acetylation (Tomikawa et al. 2012) (*Top portion*). A schematic representation of the mouse *Kiss1* gene depicts several critical regions as defined by color and shapes including a 5′ region (*black*), a coding region (*green*) that includes a promoter (*lime green box*) 3 exons (*numbered boxes*) and a CpG island (*red box*, within the third exon), a 3′ region (*blue*), and a transcriptional modifier region (*red*). *Small gray shapes* indicate sites utilized for analysis of gene conformation by Tomikawa and colleagues (2012). The table indicates the direction of H3 acetylation abundance detected in each gene region in the ARC and AVPV during E2 exposure. H3 acetylation either significantly increased (↑), decreased (↓), or remained the same (←→); N.D. = not determined (*Bottom portion*). Representations of DNA looping at the *Kiss1* gene in the ARC and AVPV. *Yellow circles* represent H3 acetylation abundance. Location of *yellow circles* enables visualization of *Kiss1* regional interactions with the acetylated histone. Prior to E2 exposure, the *Kiss1* gene structure is similar in the ARC and AVPV, though a second loop is apparent in only the ARC. E2 exposure causes formation of a complex DNA structure and decrease in *Kiss1* expression in the ARC. On the other hand, the AVPV 5′ loop is lost in favor of a long range 3′ loop and increased *Kiss1* expression during E2 exposure

would mean no net gain of histone acetylation, but a regional shift in H3 acetylation abundance. Closer examination of the histone acetyltransferase and histone deacetylase activities in kisspeptin neurons during E2 exposure will be necessary to clarify the relationship between histone acetylation and *Kiss 1* gene expression in the ARC and AVPV alike.

Based on these intriguing discoveries, critical question arises. First, how do these different populations of kisspeptin neurons evolve to create distinct orientations of the kisspeptin gene? Are there sex differences in *Kiss1* gene conformation? Also, how might DNA methylation status effect chromatin conformation? Tomikawa and colleagues found no remarkable differences in CpG methylation status, but they only assessed 6 CpG sites near the transcription start site. Importantly, the most pronounced CGI of the *Kiss1* gene is located in the third exon. There are presently no reports of differences in methylation status across this region between ARC and AVPV tissue. This could be critical, as the *Kiss1* gene contains dense CCCTC-binding factor (CTCF) sites surrounding the last exon as well as between the promoter and first exon. As CTCF is a well-described modifier of chromatin loop structure that is profoundly influenced by DNA methylation status (Ito et al. 2013; Ong and Corces 2014), it is tempting to suggest that DNA methylation could factor heavily into brain region-specific *Kiss1* gene conformations. Nevertheless, at this time, there are no reports of CTCF binding within the *Kiss1* gene or differences in methylation status across the third exon CGI.

11.8 Epigenetic Regulation of the *Kiss1* Gene and Kisspeptin Cells in the Context of Puberty

Kisspeptin signaling is integral to pubertal onset and progression. Because puberty onset is heavily influenced by environmental conditions including climate, stress, body weight, and environmental estrogens, interest in the epigenetic regulation of kisspeptin cell development and puberty-related *Kiss1* gene expression has grown substantially. As mentioned earlier, similar to the findings of Mellon and colleagues comparing *GnRH* gene promoter structure of immature and mature cell lines, Lomniczi et al. (2013) recently reported that the female rat pubertal transition is accompanied by increased prevalence of activating histone posttranslational modifications (PTMs) at the *Kiss1* promoter. Specifically, prior to puberty onset, the kisspeptin promoter was associated with a bivalent domain (i.e., H3K27me3 and H3K4me3) and occupied by a component of a polycomb repressive complex, EED. The transition to puberty was accompanied by decreased EED occupancy of the kisspeptin promoter, a gradual loss of the repressive H3K27me3 PTM, and increased levels of the permissive H3K9,14 acetylation and H3K4me3 PTMs (Fig. 11.5). The authors suggest this process is the consequence of increased DNA methylation of the EED promoter, leading to lower EED expression and consequential decreased occupancy of the kisspeptin promoter by the repressive PRC2 (EED, SUZ12, Ezh2) complex. These conclusions were largely based on observations of

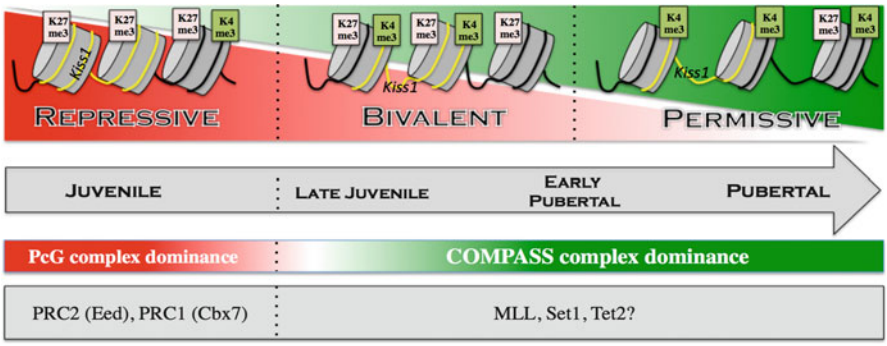

Fig. 11.5 The onset of puberty is preceded by activation of a bivalent domain near the *Kiss1* gene in the female rodent ARC. During the late juvenile period, histone 3 (H3) lysine 4 trimethylation (K4me3) accumulates near the *Kiss1* promoter in the female rate medial basal hypothalamus. This accumulation precedes loss of H3K27me3, creating a bivalent domain and an elevation of *Kiss1* gene expression. As puberty progresses, PcG complexes (e.g., PRC2 component EED) vacate the *Kiss1* promoter, presumably in favor of H3K4me3 promoting COMPASS-like complexes

dramatically delayed puberty in female mice when DNA methylation was inhibited by peripheral administration of the pharmacological DNA methyltransferase inhibitor 5-azacytidine (5-aza). While the conclusions are consistent with the observations, this 5-aza activity may not be specific to inhibition of postmitotic neuronal DNA methylation. Because the well-characterized mechanism of 5-aza requires nucleoside incorporation into DNA (Schermelleh et al. 2005; Stresemann and Lyko 2008; Svedruzic 2008), the mechanism of DNMT inhibition in postmitotic cells by this compound remains unclear. In addition, a significant reduction in growth rate and elevated levels of plasma corticosterone after the initiation of drug treatment (Lomniczi et al. 2013) is indicative of toxicities that likely contribute to delays in maturation. Because of these concerns, more direct approaches (e.g., cell-specific genetic or enzyme expression manipulations) will be necessary to clarify the relationship between DNA methylation, chromatin modifications, and puberty onset. Nonetheless, these studies are instrumental to the notion that structural modification of bivalent promoters in the neuroendocrine hypothalamus is an integral step toward puberty onset and reproductive function.

Lomniczi and colleagues' focused approach toward measuring histone modification status across development at one gene has tremendous value for clarifying the temporal progression of bivalent promoter transactivation. Importantly, they report that permissive histone PTMs (H3K4me3 and H3K9,14 acetylation) accumulate near the kisspeptin promoter during the transition from juvenile to early pubertal stages. This preceded loss of the repressive H3K27me3 PTM, suggesting that recruitment of activating complexes is imperative for transactivation and increased gene expression. Based on the temporal relationship between H3K4me3 accumulation and loss of H3K27me3, as well as the association between Tet2 and H3K4me3,

we recently evaluated the impact of kisspeptin cell-specific ablation of Tet2 activity (kTKO animals) on puberty onset and progression (Kurian et al. 2014). Remarkably, Tet2 disruption delayed puberty onset and progression in both male and female kTKO animals. Whether this impact on puberty is the consequence of Tet2-mediated DNA demethylation, DNA hydroxymethylation patterns, H3K4me3 accumulation, or another mechanism remains open and important questions. Which kisspeptin cell genomic locations, in addition to *Kiss1*, are altered by Tet2 disruption also remains unknown though likely influential.

The likeliness of additional genomic targets as integral positions of epigenetic regulation in kisspeptin cells is predicated on the recent discoveries of additional peptides that are often co-expressed in kisspeptin neurons, namely, neurokinin b and dynorphin. In fact, *tac2* (neurokinin b) expression in the ARC is an early marker of pubertal onset (Gill et al. 2012a), likely regulated by a bivalent promoter. As evidence, recent preliminary studies (Gill et al. 2012b) found that heterozygous LSD1 (a histone lysine demethylase of di- and mono-methylated H3K4) knockout female mice exhibit precocious vaginal opening and first ovulation (3–4 days prior to wild-type littermates) with early elevations of plasma gonadotropins and hypothalamic expression of *tac2* (Gill et al. 2012b). Importantly, these findings are analogous to those of Lomniczi et al. (2013), in that lower expression of an epigenetic repressive enzyme is related to elevated hypothalamic expression of a puberty-related gene. However, LSD1 expression or activity changes across typical development are currently unknown. In addition, LSD1 also stimulates hormone (ligand-associated androgen receptor)-mediated gene activation through demethylation of H3K9 (Metzger et al. 2005). Consequently, while preliminary evidence suggests the intriguing possibility that LSD1 directly alters hypothalamic development (including kisspeptin neurons) during the pubertal transition, models for cell- or region-specific genetic manipulations will be necessary to verify this interpretation. These models will be critical for determining the primary activity of LSD1 (i.e., H3K4 or H3K9 demethylation) as it relates to reproductive maturation.

11.9 Conclusion

Our understanding of epigenetic regulation of GnRH and kisspeptin neurons and reproductive neuroendocrine function in general is still in its infancy, though synthesis of the reports detailed in this chapter highlights exciting connections between epigenetic regulations in different cells of the reproductive neuroendocrine hypothalamus. The field is progressing rapidly and will undoubtedly continue its contributions to our understanding of epigenetic regulation of hypothalamic neuronal function and activity in general. With the isolation of specific mechanisms and responsible enzymes, we are inching closer to the ultimate promise of neuroendocrine epigenetic research: to define how our environments influence our physiology and behavior.

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