Epigenetics and Human Health

Dietmar Spengler
Elisabeth Binder *Editors*

Epigenetics and Neuroendocrinology

Clinical Focus on Psychiatry, Volume 2

Springer

Epigenetics and Human Health

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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 (vol 1, 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 (vol 2, Part I).

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 (vol 1, 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 (vol 1, Part I and vol 2, Parts I–III).

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 (vol 1, Part I and vol 2, Parts I–III).

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 (vol 1, 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 (vol 1, Part II). Epigenetic programming of neuroendocrine and behavioral phenotypes is sex dependent (vol 1, Parts I–II and vol 2, Parts I–III), 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 (vol 1, 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 (vol 1, 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 (vol 1, 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 (vol 2, Part III). 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 (vol 2, Part I). Moreover, the DNA methylation status at glucocorticoid receptor response elements can regulate cell-type specific enhancer activity (vol 1, 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 (vol 2, Part II). 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 (vol 2, Part III). 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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Chapter 1
FKBP5 Epialleles

Torsten Klengel and Theo Rein

Abstract Neuroendocrine regulation of the hypothalamic-pituitary-adrenal (HPA) axis relies on the fine-tuned interplay of a multitude of molecular players. The glucocorticoid receptor (GR) takes the central stage in balancing the activity of the HPA axis through its negative feedback on the peptides corticotrophin-releasing hormone and adrenocorticotropic hormone. The activity of GR, in turn, is also controlled by several cofactors including FK506-binding protein 51 (FKBP51) that emerged as potent inhibitory protein of GR. Polymorphisms of its gene *FKBP5* have been consistently linked to stress-related diseases such as major depression post-traumatic stress disorder and other neuropsychiatric phenotypes. In addition, recent studies showed that polymorphisms of *FKBP5* could also be linked to epigenetic changes evoked in response to stress exposure, giving rise to "epialleles." Here we present and discuss *FKBP5* as stress reactivity gene describing the molecular genetics and epigenetics of *FKBP5* and illustrate FKBP5 as a model at the interface of genetics, epigenetics, and neuroendocrinology.

Keywords *FKBP5* • FKBP51 • Glucocorticoid receptor • DNA methylation Major depression • Post-traumatic stress disorder

Activation of the HPA axis upon perception of stress is a prime example of the neuroendocrine regulation of the organism's reaction to environmental cues. The function of the HPA axis and its role in stress-related mental disorders has been described in detail before, and the reader is referred to excellent reviews for more details (De Kloet et al. 2005; Moisiadis and Matthews 2014a, b). Briefly, the HPA

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axis consists of the peptide corticotrophin-releasing hormone (CRH) that is secreted in the hypothalamus and triggers the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH, in turn, leads to the release of cortisol from the adrenal glands. In general, this steroid hormone executes its functions through binding to and activating the two corticosteroid receptors, namely, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). Importantly, the levels of the hormones constituting the HPA axis are balanced by the negative feedback of GR on the production and release of CRH and ACTH.

The wealth of reports documenting the link of HPA axis function to stress-related diseases has inspired many researchers to investigate the role of the molecular executors of the stress response, including the GR and its regulatory proteins. This resulted, for example, in the formulation of the corticosteroid receptor hypothesis of depression (Holsboer 2000), which stipulates impaired signaling of the corticosteroid receptors as key mechanism in the pathophysiology of depression. Further research in understanding the regulatory mechanisms governing GR activity, such as elucidating the role of chaperones and cochaperones (Wochnik et al. 2004, 2005; Grad and Picard 2007), formed the basis for the selection of HPA axis-related candidate genes for one of the first gene association study in depression and antidepressant response (Binder et al. 2004). This investigation revealed the association of polymorphisms in *FKBP5* with rapid response to antidepressants and with increased recurrence of depressive episodes and sparked intensified research efforts on *FKBP5*in psychiatric genetics, molecular biology, and beyond.

1.1 Molecular and Physiological Functions of *FKBP5*

FKBP5 is a more than 100 kb large gene (in humans) that codes for the protein FKBP51 which belongs to the family of immunophilins (Kang et al. 2008) due to its ability to bind to the immune suppressive drug FK506. FKBP51 is a co-chaperone of the central chaperone HSP90 and was originally discovered in a complex with the progesterone receptor (Nakao et al. 1985; Smith et al. 1990; Sanchez 1990). Evidence has also been provided for a genuine chaperone function of FKBP51, independently of HSP90 (Pirkl and Buchner 2001). FKBP51 features a peptidylprolyl isomerase activity located in its N-terminal domain (Sinars et al. 2003; Pirkl and Buchner 2001). To date, the biological role of this enzymatic activity of FKBP51 remains elusive. Further, FKBP51 harbors a protein interaction motif, the so-called tetratricopeptide repeat (TPR) motif that conveys the ability of FKBP51 to interact with HSP90 (Brinker et al. 2002) and thereby with HSP90-based folding complexes such as heterocomplexes of GR, chaperones, and cochaperones. Further molecular details of FKBP51 are presented in recent reviews (Storer et al. 2011).

FKBP51 was first implicated in the regulation of GR by the observation of a seeming "HPA overdrive" in squirrel monkeys (Chrousos et al. 1982, 1986). Even though these primates displayed no indications of cortisol excess, high levels of circulating cortisol have been determined; the apparent absence of physiological consequences has been ascribed to the low hormone-binding affinity of their GR (Chrousos et al. 1982, 1986). In other words, the high levels of cortisol are required

to compensate for the low affinity of GR for cortisol. This glucocorticoid resistance cannot be explained by mutations in the sequence of GR (Reynolds et al. 1997) but by the high levels of FKBP51. Different laboratories demonstrated an inhibitory action of FKBP51 on GR (Reynolds et al. 1999; Denny et al. 2000; Schülke et al. 2010; Wochnik et al. 2005; Riggs et al. 2003). FKBP51 impacts GR function on at least two levels (Fig. 1.1): its presence in the GR-chaperone heterocomplex leads to decreased binding affinity of GR and to delayed nuclear translocation (Wochnik et al. 2005; Denny et al. 2000; Reynolds et al. 1999; Tatro et al. 2009).

It is currently assumed that FKBP51 acts on GR by substituting other TPR domain proteins in the GR heterocomplex, although it cannot be excluded that still unknown specific molecular actions of FKBP51 are required (Schülke et al. 2010; Wochnik et al. 2005; Riggs et al. 2003). Meanwhile, FKBP51 has been implicated in a range of additional molecular interactions and cellular functions. Among them are other steroid receptors including MR, the kinase Akt, and its associated phosphatase PHLPP with consequences for cell proliferation and cancer treatment, the autophagy protein Beclin1, and initiation of autophagy with repercussions for antidepressant effects, microtubule polymerization, neurite outgrowth, NF-κB and immune function, and aging to name a few (Schülke et al. 2010; Jinwal et al. 2010; O'Leary et al. 2011; Pei et al. 2009; Gaali et al. 2015; Gassen et al. 2014; Quinta et al. 2010; Storer et al. 2011).

An important feature of the *FKBP5* gene, which is also relevant for the later discussion of the *FKBP5* epialleles, is its inducibility by glucocorticoids and other steroids that has long been known (Reynolds et al. 1998; Baughman et al. 1997; Harrigan et al. 1989). Intronic regions of *FKBP5* feature functional glucocorticoid responsive elements (Hubler and Scammell 2004), and like with many other genes, long-distance interactions are operative in the GR-dependent control of *FKBP5* transcription (Paakinaho et al. 2010; Klengel et al. 2013). The GR dependency of *FKBP5* mRNA production represents an ultrashort intracellular feedback loop that complements the systemic feedback loop of the HPA axis in balancing the stress response. This intracellular feedback loop needs also to be taken into consideration in the interpretation of animal experiments using *Fkbp5* gene knockout mice to establish the role of FKBP51 in shaping the physiological stress response (Touma et al. 2011; Hartmann et al. 2012; Hoeijmakers et al. 2014). The effect of *Fkbp5* knockout was most pronounced after repeated or strong stressors, which could be explained by the production of additional FKBP51 in wild-type but obviously not in knockout mice in response to these stressors.

1.2 Epigenetic Regulation of *FKBP5*

1.2.1 Epigenetic Memory Through DNA Methylation

Although valuable discussions about the definition of epigenetics are ongoing, we will follow the definition of epigenetics as the study of chromatin changes that alter genome activity (and thereby also cellular and physiological traits) without changing the DNA sequence, irrespective of the debate of heritability. This is important in particular for nondividing, postmitotic cells such as neurons where epigenetic

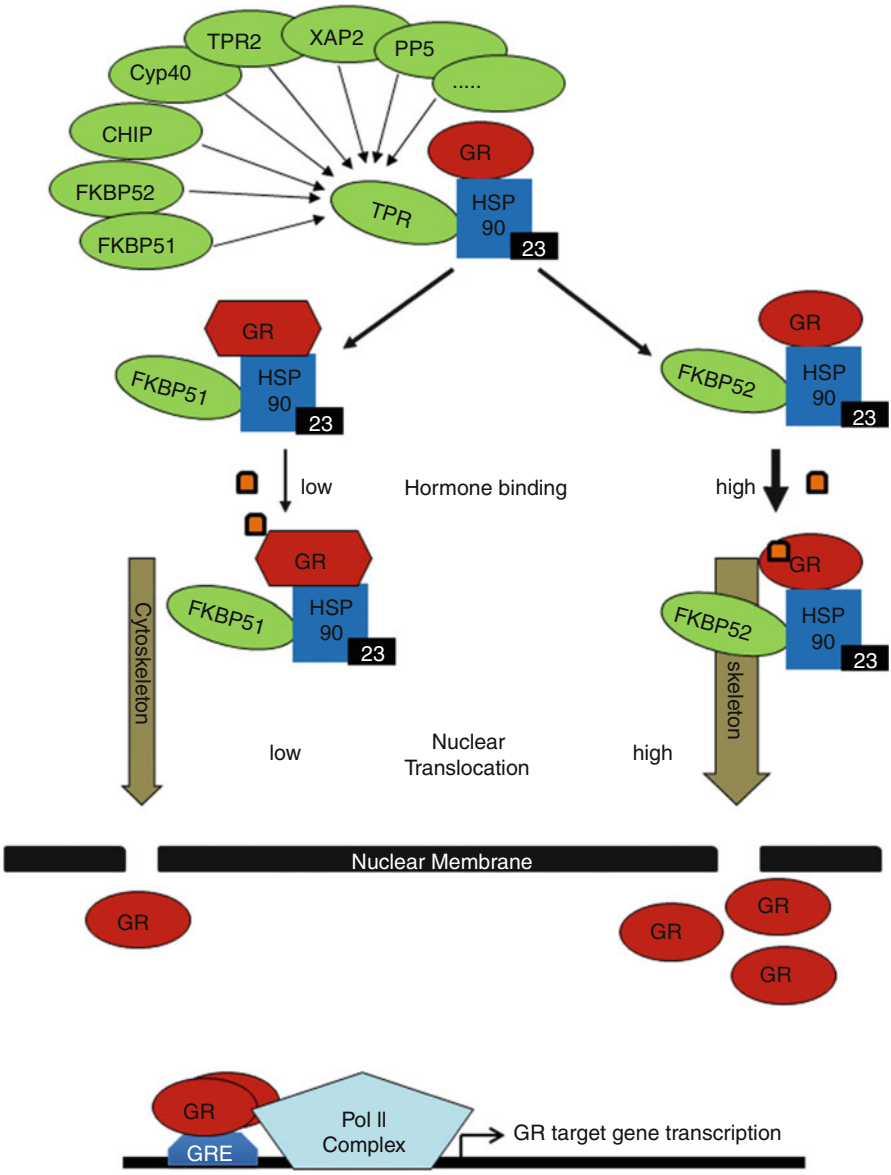

Fig. 1.1 Regulation of GR activity by FKBP51 and competing TPR proteins. In the absence of hormone, GR resides in the cytosol in a chaperone heterocomplex that is composed of Hsp90, p23, one of the cochaperones featuring a TPR (tetratricopeptide repeat) domain and possibly other cochaperones. The TPR proteins competitively integrate into the heterocomplex (Schülke et al. 2010). When FKBP51 is present in the GR heterocomplex, GR hormone-binding affinity is low, most likely due to a different GR conformation. Since FKBP51 does not mediate association with the motor protein complex, nuclear translocation of GR is delayed. In the presence of appropriate TPR proteins such as FKBP52, GR efficiently translocates to the nucleus upon hormone binding, interacts with glucocorticoid responsive elements (GREs), and engages in chromatin remodeling and activation of the polymerase II transcriptional complex to drive target gene transcription

regulation of gene expression can be detected in response to environmental conditions. In the following section, we will focus on epigenetic regulation through DNA methylation as reversible epigenetic modification that can be studied by multiple techniques on a genome-wide level in larger human cohorts.

DNA methylation in mammals refers to the chemical modification of cytosines by a methyl group. Here, DNA methylation occurs predominantly, but not exclusively, at cytosines within CG dinucleotides. Highly methylated DNA is detectable at centromeric DNA and repetitive elements but also to various degrees at regulatory elements such as promoters and enhancer/repressor regions, controlling genome stability, cell type lineage specification, and gene expression in rodents and human (Jones 2012). The principles on how regular epigenetic marks are established in a spatiotemporal manner are incompletely understood. Nevertheless, the distribution of methylation marks across the genome and different cell types argue in favor for a highly controlled process (Schübeler 2015). The genetic scaffolding and protein binding to it at least in part guide the process (Stadler et al. 2011; Lienert et al. 2011). Methylation profiles tend to cluster with regions that are not methylated (e.g., most CpG islands) and highly methylated (e.g., repetitive elements) and regions that are low to intermediately methylated. The latter seem to be of particular relevance for transcriptional regulation since many regulatory regions such as enhancers fall into this category (Stadler et al. 2011). The loss of methylation can occur passively during cell division but can also be actively catalyzed, i.e., through the ten-eleven translocation (TET) proteins. We would like to refer the reader to specialized reviews on the regulation of DNA methylation and demethylation for further reading (Bhutani et al. 2011; Robertson 2005; Laird 2003; Berger 2007; Pastor et al. 2013).

1.2.2 Imprinted Loci, Metastable Epialleles, and Allele-Specific Methylation Changes

In mammals, about 1 % of all genes are expressed from either the maternal or the paternal allele, a phenomenon termed genomic imprinting (Reik and Walter 2001). This parent-of-origin effect is achieved by methylation and thus imprinting of the paternal allele in the germline leading to an exclusive expression from the maternal allele or vice versa. A classic example of a dysregulation of this type of regulation is the Prader-Willi/Angelman syndromes. The paternal deletion of a region on chromosome 15 leads to Prader-Willi syndrome by the loss of several genes that are exclusively expressed from the paternal allele with the maternal alleles silenced through imprinting. In turn, the Angelman syndrome is caused by the deletion of the maternal allele with genes on the paternal alleles silenced through imprinting. Thus, the correct location of methylation-guided gene expression silencing is crucial with regard to human disorders related to genomic imprinting (Horsthemke and Wagstaff 2008).

The term imprinting is also used more loosely with regard to other phenomena that may be related to genomic imprinting. While genomic imprinting typically refers to the epigenetic modification of parental allele in the germline, imprinting in a broader sense is used to describe lasting changes in epigenetic modifications in somatic cells as well. An example is metabolic imprinting or programming, the epigenetic regulation of metabolic processes in response to the availability of nutritional supplies in the prenatal and early postnatal period (Tang and Ho 2007; Vickers 2014). These early developmental stages offer ample plasticity not only in genes and pathways regulating metabolic processes. Chromatin modifications in general may integrate the transcriptional response to environmental signals and relay them subsequently to a long-lasting altered gene expression profile thus providing mechanisms for the cross talk of the environment with the genetic blueprint. With respect to psychiatric disorders and neuroendocrine regulation, it has long been understood that environmental stressors have a profound impact on the pathophysiology of stress-related disorders (Heim and Nemeroff 1999; Kessler et al. 1997). Efforts in the past years have emphasized the relative contribution of the innate genetic predisposition toward psychiatric disorders (Sullivan et al. 2012), but more recently statistical as well as molecular frameworks emerge to combine both aspects in gene by environment interaction studies (Manuck and McCaffery 2014). The influence of the environment can start as early as before conception and has implications for disease development to a varying degree in different developmental stages from in utero and early childhood to adulthood (Provencal and Binder 2014).

Important in the discussion about gene by environment interaction is the term "epialleles" or "metastable epialleles." Epialleles are defined as variations of the same gene or genetic locus that differ in their epigenetic endowment and thus activity but not in their DNA sequence (Dolinoy et al. 2007; Finer et al. 2011). An example is the murine yellow agouti allele (A^{vy}) that results in a yellow to brown coat color as function of varying degrees of DNA methylation at a retrotransposon element (Waterland and Jirtle 2003). Notably, the methylation levels are sensitive to dietary supplementation with methyl donors. The definition of epialleles is usually not followed in a strict sense as the term "epialleles" can also be applied to alleles that slightly differ in DNA sequence, frequently in the case of single nucleotide polymorphisms that do not change protein coding but gene activity through mechanisms that involve epigenetic alterations. Although current evidence might underestimate the influence of small genetic variations on DNA methylation, these small differences could account for a large portion of epigenetic differences observed in current studies as discussed above (Bell et al. 2011; Fraser et al. 2012).

An example relevant to stress physiology and depression is the allele-specific programming of neuropeptide Y (NPY) in a genetic mouse model of depression (Melas et al. 2013). NPY is widely expressed in the brain and has been implicated in the stress response, emotional processing, and depression (Mickey et al. 2011; Cohen et al. 2012). In humans, SNPs in NPY consistently have been associated with depression (Bosker et al. 2011; Mickey et al. 2011). In rats, an SNP in the promoter (C/T, rs105431668) has been identified that differentiates a genetic model of depression (called "Flinders sensitive line") from controls. This SNP impacts on DNA

interaction of the transcription factor CREB2 and of the histone acetyltransferase Ep300, thereby also affecting mRNA levels of NPY and acetylation of histone 3 at lysine 18. Thus, this SNP appears to change the epigenetic landscape of the NPY promoter or, in other words, lead to different epialleles of NPY, which further might be the basis of the differences in depression-like behavior. In this model, the epialleles are SNP directed, like in the case of *FKBP5* we present in the following section.

1.3 *FKBP5* Epialleles

Polymorphisms in *FKBP5* were associated with rapid response to antidepressants and with increased recurrence of depressive episodes (Binder et al. 2004), and the so-called risk allele of rs1360780 has been shown to lead to increased cortisol levels in response to stress in healthy individuals (Ising et al. 2008). This has been corroborated in infants by the finding of increased salivary cortisol levels in response to a mild stressor (Luijk et al. 2010). The *FKBP5* risk allele not only affects the endocrine system but also is associated with functional MRI outcomes in form of an attention bias to threat (Fani et al. 2013). With regard to psychiatric disorders, mixed results have been obtained. In individuals with major depression, risk allele carriers exhibit GR resistance, i.e., reduced suppression of the HPA axis in response to oral administration of dexamethasone. In parallel, the induction of *FKBP5* mRNA in peripheral blood is reduced in MDD patients carrying the risk allele (Menke et al. 2013). On the other hand, contrasting results have been obtained in PTSD patients. Here, risk allele carriers show enhanced GR sensitivity (Binder et al. 2008; Mehta et al. 2011).

In addition to main genetic effects of the *FKBP5* risk allele haplotype, *FKBP5* became a prime example for gene by environment interaction on psychiatric phenotypes. Here, the interaction of early life stress events, mostly in the form of childhood physical and sexual abuse, with *FKBP5* results in the precipitation of post-traumatic stress disorder (PTSD), major depressive episodes, and other psychiatric (endo)phenotypes (Binder et al. 2004, 2008; Zimmermann et al. 2011; Xie et al. 2010; Bevilacqua et al. 2012; Appel et al. 2011; Roy et al. 2010; Boscarino et al. 2012; White et al. 2012; Dackis et al. 2012; Collip et al. 2013; Lessard and Holman 2014) (see Table 1.1).

Epigenetic changes have received increased attention in neuroscience as mediators of environmental factors acting on brain function in humans and in rodent animal models with a strong emphasis on stress-related phenotypes (Weaver et al. 2004; Petronis 2010; McGowan et al. 2009; Murgatroyd et al. 2009). Moreover, epigenetic mechanisms may explain the molecular underpinnings of gene by environment interactions integrating the mutual interaction of the genetic blueprint with environmental conditions on the risk to develop psychiatric disorders. As an example, we have shown such a potential mechanism linking *FKBP5* polymorphisms and exposure to childhood abuse on the risk to develop PTSD (Klengel et al. 2013). We will summarize these findings in the next section.

Table 1.1 Summary of the main results of the here surveyed studies examining the effects of FKBP5 polymorphisms

Study	Year	Genetic variant investigated	Main results
Binder et al.	2004	rs1360780	Significant association of response to antidepressants and the recurrence of depressive episodes, increased intracellular FKBP5 protein expression in T-allele carrier
Ising et al.	2008	rs4713916, rs1360780, rs3800737	Incomplete recovery of the cortisol secretion after the Trier Social Stress Test, increased self-reported anxiety in minor allele carrier
van Luijk et al.	2010	rs1360780	Higher cortisol reactivity in infants with minor T-allele during Strange Situation Procedure
Fani et al.	2013	rs1360780	T-allele carrier shows attention bias toward threat and increased hippocampal activation during behavioral response on a dot-probe task and hippocampal activation during task performance
Menke et al.	2013	rs1360780	Reduced FKBP5 transcriptional activation in MDD patients carrying the T-allele with a reduced cortisol suppression
Binder et al.	2008	rs9296158, rs3800373, rs1360780, and rs9470080	Interaction of the exposure to childhood maltreatment and FKBP5 polymorphisms on the PTSD severity in adulthood, minor allele carrier exposed to childhood abuse shows more severe PTSD symptoms in adulthood
Mehta et al.	2011	rs9296158	Minor A-allele carrier shows GR super sensitivity with PTSD; baseline cortisol levels were decreased in PTSD in patients with the GG genotype; in addition, FKBP5 genotype influences gene expression profile in individuals with PTSD
Zimmermann et al.	2011	rs9296158, rs3800373, rs1360780, rs4713916, and rs9470080	Risk alleles interact with lifetime trauma on the development of depressive episodes
Xie et al.	2010	rs9296158, rs3800373, rs1360780, and rs9470080	For rs9470080 risk allele carrier had a higher risk to develop PTSD in response to early trauma exposure
Bevilacqua et al.	2012	rs9296158, rs3800373, rs1360780, and rs9470080	Except for rs9296158, risk alleles interact with childhood abuse on increased aggression in adulthood

Table 1.1 (continued)

Study	Year	Genetic variant investigated	Main results
Appel et al.	2011	rs1360780	Risk allele interacts with exposure to childhood abuse on the risk to develop depressive episodes in adulthood
Roy et al.	2010	rs9296158, rs3800373, rs1360780, rs4713916, rs377747, and rs9470080	For rs9296158, rs3800373, and rs1360780, risk alleles interact with childhood abuse on the risk of suicide attempts in adulthood
Boscarino et al.	2012	rs9470080	The combination of Fkbp5 and other risk genes interacts with early trauma on lifetime PTD and early onset PTSD
White et al.	2012	rs9296158, rs3800373, rs1360780, rs4713916, rs377747, and rs9470080	Risk alleles interact with emotional neglect in childhood on increased dorsal amygdala reactivity
Dackis et al.	2012	rs9296158, rs3800373, rs1360780 haplotype	Risk haplotype interacts with childhood trauma on limbic irritability
Collip et al.	2013	rs9296158, rs1043805, rs4713916, rs1360780	rs9296158, rs1043805, and rs1360780 risk alleles interact with childhood trauma on psychotic symptoms
Lessard and Holman	2014	rs1360780	Childhood and adult stress interact with T-allele on physical health
Klengel et al.	2013	rs1360780	T-allele carrier exposed to childhood trauma shows a reduced FKBP5 methylation compared to protective genotype carrier
Paqette et al.	2014	rs1360780	T-allele carrier showed highest FKBP5 expression in the placenta, which was negatively correlated to FKBP5 DNA methylation
Hoehne et al.	2014	rs1360780	Healthy controls but not individuals with remitted MDD showed highest cortisol levels when carrying the T-allele with a blunted FKBP5 transcriptional response to psychosocial stress

FKBP5 SNPs implicated in the genetic association studies but also in the gene by environment studies are distributed across the locus spanning more than 100 kb of genomic sequence. These SNPs are in high linkage disequilibrium and located mostly in intronic regions. Thus, it is challenging to determine the actual functional

polymorphism. Several studies mentioned above implicated in particular rs1360780 as a modulator of the genetic effects. This SNP is located close to a glucocorticoid response element (GRE) in intron 2 that has been reported to drive *FKBP5* gene expression (Paakinaho et al. 2010). The proximity of this SNP to a functional GRE was of particular interest, because activated GR has been shown to induce lasting epigenetic changes that shape subsequent responses to glucocorticoids (Kress et al. 2006; Thomassin et al. 2001). Further, the SNP and the nearby GRE are located about 50 kb from the transcriptional start site, and chromatin looping has been suggested to enable communication of the distant DNA regions and promotion of transcription from a distant GRE (Paakinaho et al. 2010).

Reporter gene analyses showed that the intronic SNP rs1360780 affected both the non-stimulated and the glucocorticoid-stimulated transcriptional activity of the DNA stretch encompassing the SNP and the GRE (Klengel and Binder 2013). This was accompanied by genotype-dependent binding of the TATA-binding protein in vitro resulting potentially from the formation of a TATA box-like sequence by the T-allele of the SNP. This may in addition recruit proteins that facilitate the transcriptional activation of FKBP5 by this enhancer region. In addition, the SNP leads to allele-specific chromatin loops that link sequences of intron 2 with a DNA stretch in intron 7 and with the transcriptional start site as determined by chromatin conformation capture (3C) experiments (Klengel and Binder 2013). These data support the notion that rs1360780 indeed represents the functional SNP influencing the genetic predisposition to an enhanced transcriptional activation of *FKBP5* in response to stress through an increase of the intron 2 enhancer function and a chromatin looping of the enhancer region to the transcription start site.

Subsequent DNA methylation analysis revealed that the SNP rs1360780 is not only a genetic predisposition for an enhanced transcriptional response but also moderates the epigenetic response of *FKBP5* to childhood abuse through an allele-specific *FKBP5* demethylation. Intriguingly, the demethylation affected intron 7 that was linked with intron 2 (harboring the rs1360780 SNP) through a chromatin loop in an allele-specific manner. Reporter gene assays confirmed that the intron 7 GREs drive glucocorticoid-dependent transcription, which is affected by DNA methylation. The genetic variant thus drives the epigenetic embedding of early life trauma with the risk allele leading to an alteration of the DNA methylation in intron 7 and subsequent amplifying of the transcriptional response to stress. In contrast, individuals with the protective genotype retain their DNA methylation pattern in intron 7.

These findings can be summarized in the following model that is depicted in Fig. 1.2 (Klengel and Binder 2013; Klengel et al. 2013): The intronic SNP rs1360780 directs GR binding, chromosomal conformation, and transcriptional activation of *FKBP5*, all in an allele-specific manner. This SNP also governs demethylation in intron 7 in an indirect manner through chromatin looping which enables communication between intron 2 chromatin factors and intron 7. This model explains the allele-specific gene-environment interactions of *FKBP5* and early life stress. The cortisol reactivity of *FKBP5* is programmed by early life stress by the SNP-dependent and allele-specific demethylation around the intron 7 GREs. In principle,

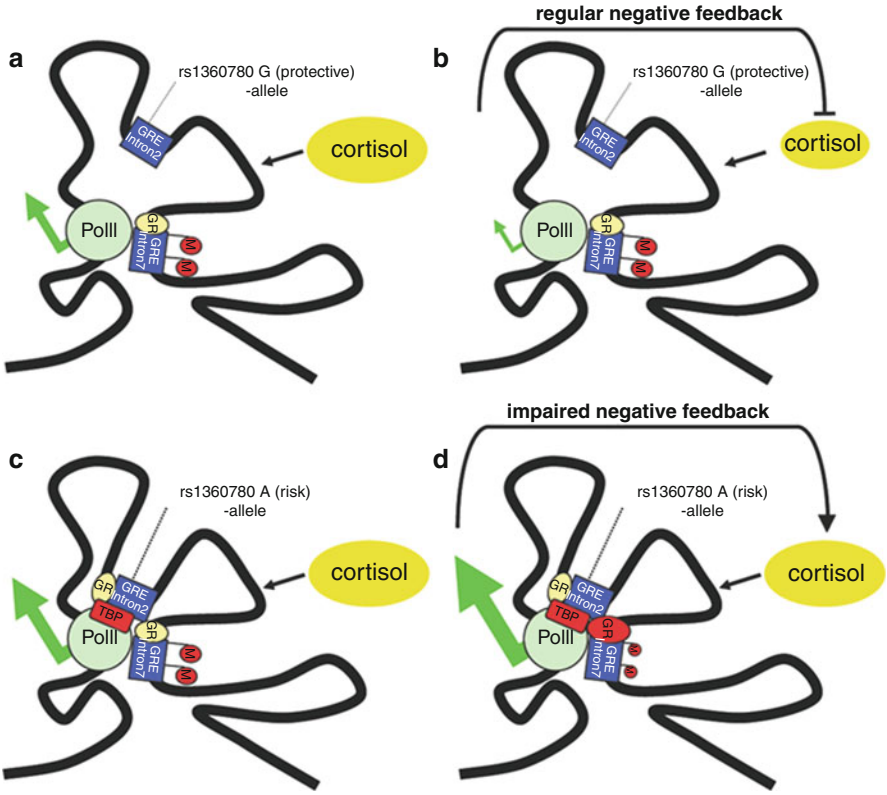

Fig. 1.2 Model of trauma induced allele-specific demethylation of FKBP5 through long-distance interactions. This figure is replicated from Klengel et al. 2013. (**a**) *FKBP5* mRNA transcription is induced by cortisol via a three-dimensional interaction and loop formation of predominantly distal enhancer regions harboring GREs that are located in the promoter region, intron 2, 5, and 7 with the core promoter site. Only interactions confirmed by three-dimensional interaction analysis are depicted by *blue boxes*. The GR as activating transcription factor is represented in *light yellow* (PolII = RNA Polymerase II). (**b**) When the acute stressor is over, a negative feedback mechanism via the GR leads to the normalization of cortisol levels in carriers of the G (protective) allele of rs1360780 and in consequence less transcriptional effects on GR target genes, including *FKBP5* (*FKBP5* transcription is represented by *green arrows*). (**c**) The A (risk) allele of rs1360780 confers a stronger interaction of intron 2 with the promoter region and is associated with an increased binding of TATA box-binding protein (TBP, represented in *red*), potentially by formation of an additional TATA box-like sequence in intron 2. When this enhancer region is looped to the promoter site, the additional TBP binding may facilitate increased recruitment of transcription factors and finally RNA polymerase II. This leads to a stronger transcriptional induction by cortisol in carriers of the risk allele vs. carriers of the protective genotype as depicted in panel (**a**) and indicated by the *green arrow*. In risk allele carriers, the initial rise in cortisol and GR activation leads to higher levels of FKBP51, which have been associated with an impaired negative feedback of the stress hormone axis and prolonged elevations of cortisol levels after a single stressor. (**d**) This long-lasting cortisol exposure then induces DNA demethylation around functional GREs in intron 7 (depicted by *red* 'M' *circles*). This further enhances the genetic predisposition for a stronger GR-induced transcription of *FKBP5* (stronger GR activation in intron 7 represented by the *red* GR element), leading to changes in GR sensitivity (Figure and figure legend taken from Klengel et al. 2013)

it cannot completely be ruled out that allele-specific demethylation already existed before trauma exposure and thus would convey vulnerability to trauma. However, the observation that the demethylation increases with trauma severity strongly argues against this scenario. In any case, the mechanism does not involve pure epialleles in a strict sense but a hybrid situation with the SNP producing a genetically driven epigenetic allele. We propose that this type of mechanism will be relevant for gene-environment interactions in psychiatric disorders in general, as well as other diseases (Meaburn et al. 2010; Cowper-Sal et al. 2012).

Epialleles of *FKBP5* have also been reported in a recent study that determined rs1360780 genotype, *FKBP5* expression, and *FKBP5* methylation in intron 7 in the placenta of 61 infants (Paquette et al. 2014). Expression of *FKBP5* was negatively associated with DNA methylation in a genotype-dependent fashion, and carriers of the TT genotype displayed the highest expression. Since these alterations also could be related to differences in neurobehavior, the authors speculate that this interplay of genetic and epigenetic differences may shape the cortisol response in the placenta (Paquette et al. 2014). Genotype-dependent expression of *FKBP5* in relation to DNA methylation at intron 7 has also been investigated after psychosocial stress in individuals with a positive ($n=61$) and negative ($n=55$) lifetime history of major depression (Höhne et al. 2014). Interestingly, genotype-dependent regulation of *FKBP5* expression could be observed in healthy controls but not in remitted depression. The effect of DNA methylation did not reach significance in this study (Höhne et al. 2014) (see Table 1.1).

In general, noncoding regions of the genome harboring regulatory potential recently gained more attention also with regard to the initial perplexity about the discovery of disease-associated SNPs in seeming genome "deserts." This confusion has been substituted by the thriving development of concepts taking long-distance gene regulation into account. Even though epigenetics typically involves covalent modifications of DNA and histones, it appears worth taking conformational isoforms into account when defining epialleles. It is known that the epigenetic landscape is intertwined with the genetic endowment, which includes SNPs and DNA binding factors that bring about long-range chromatin interactions (Stadler et al. 2011; Schalkwyk et al. 2010; Lienert et al. 2011).

It should also be noted that in the case of the *FKBP5* intracellular communication between the epialleles is expected to occur, which should be relevant in the heterozygous situation (Fig. 1.3). The communication is brought about by the ultrashort intracellular feedback loop that consists of the activation of *FKBP5* transcription by GR that produces enhanced levels of FKBP51 which, in turn, reduce GR activity. In a situation of heterozygosity with respect to the SNP rs1360780, the allele with lower GR responsivity, which is also the "risk" allele for the development of PTSD (Klengel and Binder 2013; Klengel et al. 2013), will produce little FKBP51 in response to GR activation under, for example, stressful conditions. Even though the "protective" allele exhibits higher GR responsivity and thus produces more FKBP51 in response to GR activation, the overall level of FKBP51 is expected to be lower than in the case of two GR-responsive alleles in the cell. The lower levels of the GR inhibitor FKBP51 should result in higher GR activity. Thus, the programming

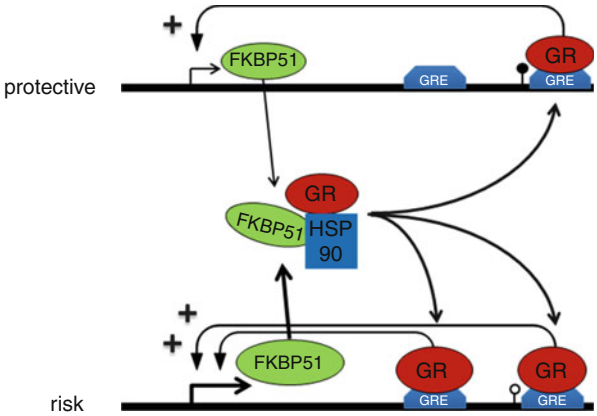

Fig. 1.3 Intracellular FKBP5 epiallele cross talk. The scheme considers the situation of two different alleles (with respect to rs1360780). *FKBP5* expression (*top*) from the allele with the "protective" genotype is stimulated through binding of the GR to GREs in intron 7 but not intron 2. FKBP51 protein leads to the reduction of the GR sensitivity to cortisol reducing the effects of GR activation thus forming an ultrashort feedback loop. On the "risk" allele (*bottom*), GR stimulates *FKBP5* expression through binding to GREs in intron 2 and intron 7 leading to a stronger transcriptional activation in comparison to the allele with the protective genotype. Furthermore, GR occupancy entails demethylation at intron 7 in the "risk" allele giving rise to two different *FKBP5* epialleles in the cell. The higher production of FKBP51 from the "risk" allele desensitizes GR and thus further decreases *FKBP5* transcription from the "protective" allele

on the GR-responsive allele through the epigenetic mechanisms detailed above is expected to be more pronounced in the presence of only one than in the presence of two GR-responsive alleles. This interdependency of the *FKBP5* (epi)alleles should not only impact on the calibration of GR activity but also could have repercussions on the HPA axis settings, even though the outcome is difficult to predict in feedback systems.

1.4 Perspectives

The vast majority of diseases-associating SNPs map to noncoding regions of the genome and thus do not alter the protein sequence (Abecasis et al. 2010; Frazer et al. 2009). The successful charting of regulatory chromatin domains including the epigenetic marks (Ram et al. 2011; Ernst et al. 2011; Heintzman et al. 2007, 2009) will greatly facilitate the efforts to map the SNPs to regulatory regions. Nevertheless, since each associated SNP has hundreds of linked SNPs and through chromatin looping might affect several genes across longer distances, we still face highly complex regulatory networks that are challenging to disentangle to pinpoint the disease-relevant molecular mechanisms. More recent computational methods may help to prioritize the putative causal SNPs (Cowper-Sal et al. 2012).

Furthermore, to move from association closer to causality, advanced methods in gene editing such as the CRISPR/CAS9 system (Hsu et al. 2014) may be helpful. Through genome editing, the creation of cellular models differing only in the SNP rs1360780 will be a suitable way to better understand the specificity of the *FKBP5* genetic alleles and epialleles. For *FKBP5*, these techniques could also answer the question whether the demethylation in intron 7 depends on the chromatin conformation or whether enhanced activity alone is sufficient. However, higher accuracy and reduced off-target modification in both genome editing and epigenome editing tools either through CRISPR/Cas9 or TALEs need to be achieved first. Another challenge in elucidating the mechanisms governing the programming of the *FKBPp5* epialleles is the delineation of the factors that determine the window of sensitivity. Here, longitudinal studies involving suitable animal models or human cohorts at risk can answer questions regarding the timing of early life stress and subsequent molecular epigenetic modifications.

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Chapter 2
Genomic Regulation of the PACAP Receptor, PAC1, and Implications for Psychiatric Disease

Kristina B. Mercer and Kerry J. Ressler

Abstract Impairment of the hypothalamic-pituitary-adrenal (HPA) axis and related neurological signaling has been attributed to several psychiatric conditions including unipolar and bipolar depression and posttraumatic stress disorder (PTSD). Consequently, irregularities in the mRNA expression or protein levels of neuropeptide hormones and receptors involved in related stress pathways can trigger these neurological disorders. As a critical modulator of the stress and fear pathways in concert with the pituitary adenylate cyclase-activating peptide (PACAP) ligand, the PAC1 receptor (PAC1) has been implicated in risk for PTSD. Genetic variants, epigenetic alterations, and hormone regulation have been attributed to changes in the expression of *ADCYAP1R1* which encodes the PAC1 protein. The chapter will focus on a review of PACAP-induced cellular function, localization, and expression of the PAC1 receptor. The goal of this review is to address the effects of altered expression of PAC1 on phenotypic outcomes, particularly those neurological in nature. We also discuss existing and potential mechanisms that can induce changes in *ADCYAP1R1* transcript levels, including genetic and epigenetic alterations and hormone regulation.

Keywords Epigenetics • Gene expression • Genetic variants • DNA methylation Splice isoforms • Estradiol

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2.1 Introduction: PACAP/PAC1 Is a Biologically Crucial Signal Involved in Various Cellular Processes in the Central Nervous System and Peripheral Tissues

Named for its ability to activate adenylate cyclase and increase production of cyclic adenosine monophosphate (cAMP) within rat pituitary cells, the pituitary adenylate cyclase-activating polypeptide, or PACAP, was first identified among protein extracts of ovine hypothalamus (Miyata et al. 1989). Sequencing of this protein isolate revealed a 38-amino-acid peptide, also known as PACAP38 to distinguish it from the smaller functional peptide PACAP27. PACAP27 is comprised of the first 27 N-terminal amino acids of PACAP38 and is the result of protein cleavage of the larger protein isoform (Miyata et al. 1990). PACAP38 and PACAP27 exist in different proportions dependent on tissue type and in brain tissue specifically. PACAP38 is the more prominent of the two protein forms (Arimura et al. 1991).

Testament to its importance in biological function, the N-terminal amino sequence shared by PACAP38 and PACAP27 is remarkably conserved across several species from protochordates to mammals (>85 % identity) (Sherwood et al. 2000). Within species, protein-protein homology has also been observed and has provided clues to PACAP function. Characterized as a member of the vasoactive intestinal peptide/secretin/glucagon superfamily, sharing similar amino acid structure and a role in cellular processes, PACAP has been identified as a regulator of nervous, endocrine, cardiovascular, muscular, and immune systems (Vaudry et al. 2009). Within the central nervous system in particular, PACAP plays a critical role as a neurotransmitter peptide involved in neurogenesis and neuroprotection (Reglodi et al. 2011). Similar to other members of its protein superfamily, PACAP is a hormone ligand that mediates regulation of these various systems through binding to guanine nucleotide-binding protein (G protein)-coupled receptors (Segre and Goldring 1993).

PACAP38 and PACAP27 are ligands for three G protein-coupled receptors, two (VPAC1 and VPAC2) of which also bind to vasoactive intestinal peptide (VIP) and the other one (PAC1) binds with highest affinity to PACAP itself (Shivers et al. 1991; Laburthe and Couvineau 2002). These three receptors are class B (or class II) G protein-coupled receptors (GPCRs) of the secretin receptor family. Similar in general function and protein structure to all other GPCRs, this family of receptor proteins contains a seven-transmembrane domain, extracellular loops enabling ligand binding, and intracellular protein domains that facilitate the transduction of signaling cascades. These receptors allow for extracellular, molecular cues to stimulate processes within the cell though interaction with specific G proteins (Rosenbaum et al. 2009). Class B GPCRs are classified as such based on sequence homology and binding to structurally related hormone ligands. PACAP-stimulated G protein-coupled receptors are responsible for the activation of adenylate cyclase (AC) or phospholipase C (PLC). Interestingly, the potency of PACAP38 or PACAP27 for activation of either signaling pathway is dependent on type of receptor isoform (Pantaloni et al. 1996).

2.2 PAC1 Receptor Isoforms Provide Flexibility in Ligand Specificity and Downstream Signaling

The gene *ADCYAP1R1* encodes for the PAC1 receptor protein (Stoffel et al. 1994). Several splice variants within this gene have been identified and are responsible for generating functional differences in the receptor protein domains (Spengler et al. 1993; Dautzenberg et al. 1999). These differences affect ligand-binding specificity, downstream signaling pathways, and other physiological outcomes. The *ADCYAP1R1* mRNA results in a protein with an extracellular N-terminal signal peptide (EC1), three extracellular loops (EC2, EC3, EC4), a seven-transmembrane region, three intracellular loops (IC1, IC2, IC3), and an intracellular C-terminal domain (Blechman and Levkowitz 2013). The N-terminal peptide region is necessary for ligand binding and the extracellular loops infer ligand-binding specificity and affinity (Harmar 2001). Likewise, the intracellular loops of the protein are integral to the type of G protein that will couple and, hence, the signaling pathway that will be activated. G proteins of the type G_s and G_q have both been implicated in PACAP-/PAC1-induced signaling pathways activating adenylate cyclase (AC) and phospholipase C (PLC) signaling cascades, respectively (Dickson and Finlayson 2009; Vaudry et al. 2009). Activation of AC by G_s results in cAMP-dependent activation of protein kinase A (McCulloch et al. 2002). Alternatively, G_q activates the PLC cascade, resulting in cleavage products, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG is responsible for the activation of protein kinase C (PKC) and IP_3 stimulates increased cytosolic calcium (Basille et al. 1995; Harmar 2001) (Fig. 2.1).

Splice isoforms that result in changes in receptor affinity for PACAP and intracellular binding of G protein type involve variations in the EC1 and IC3 loop domains, respectively (Ushiyama et al. 2007). Alternative transcripts that result from the presence or absence of 81 or 84 nucleotide cassettes (encoded by exons 14 and 15) within the IC3 loop have been characterized (Spengler et al. 1993). Five isoforms can be generated from splicing of the mRNA transcript: no cassettes isoform or null isoform (exons 14 and 15 excluded), hip isoform (exon 14 included; exon 15 excluded), hop1 isoform (exon 15 included; exon 14 excluded), hop2 isoform (an 81 nt exon 15 included; exon 14 excluded), or hip hop 1 isoform (exon 14 included; exon 15 included) (Fig. 2.2).

Alternatively spliced isoforms involving the genomic regions of the EC1 domain also exist. These isoforms, which are coined the names PAC1 short (PAC1s) and PAC1 very short (PAC1vs), lack 21 amino acids (exons 5, 6 excluded) or 57 amino acids (exons 4, 5, and 6 excluded) from the EC1 region, respectively (Dautzenberg et al. 1999) (Fig. 2.2). A normal isoform (PAC1n) that contains all three exons (4, 5, and 6) has also been identified. Each of these isoforms binds with differential affinity to PACAP and VIP, PAC1n having the highest and most selective binding for PACAP. The short receptor form results in high but nonselective affinity for both PACAP and VIP. The very short form binds preferentially to PACAP over VIP but with much lower affinity than normal isoform for either ligand (Dautzenberg et al. 1999).

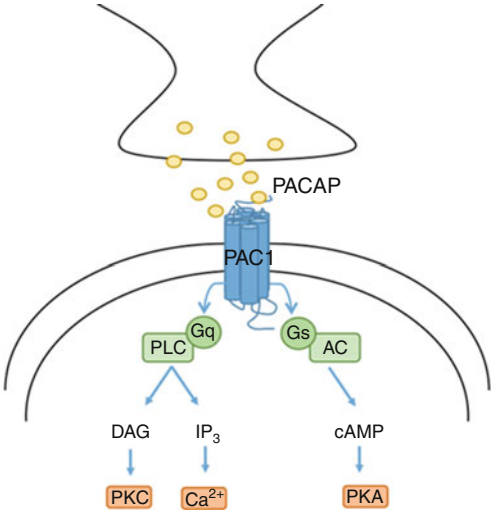

Fig. 2.1 PACAP-PAC1 signaling. PAC1 is a G protein-coupled, seven-transmembrane receptor protein that binds to PACAP within neurons. Ligand binding results in a conformational change in PAC1 which activates either G proteins Gq or Gs, stimulating phospholipase C (PLC) or adenylate cyclase (AC) and triggering signaling cascades within the cell. The PLC signaling cascade results in the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), activating protein kinase C (PKC) and increasing intracellular calcium (Ca^{2+}). The AC signaling cascade results in cAMP-dependent activation of protein kinase A (PKA). (This schematic diagram is adapted from Vaudry et al. (2009))

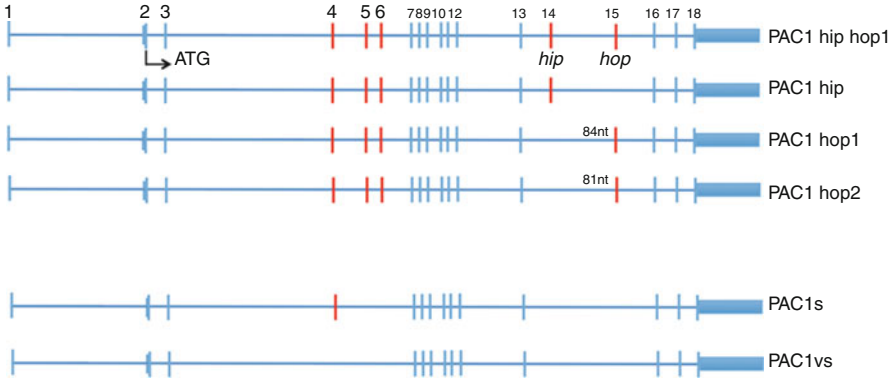

Fig. 2.2 Alternative splicing of the PAC1 gene, *ADCYAP1R1*. Alternative splicing of *ADCYAP1R1* results in several different isoforms, some of which are shown here. Exons 14 and 15, which are called the hip and hop cassette, respectively, are alternatively spliced. Transcript isoforms containing only exon 14 are called PAC1 hip. There are two variations of the PAC1 hop isoform, both of which lack exon 14 (in *red*) but contain exon 15 (in *red*) sized at either 84 or 81 nucleotides (PAC1 hop1 vs. PAC1 hop 2). Exons 4, 5, and 6 (shown in *red*) encode for amino acids within the N-terminal region of the PAC1 protein. The PAC1s transcript contains exon 4 but neither exon 5 or 6. The transcript PAC1vs does not inlcude exons 4, 5, or 6. Both exons 14 and 15 are also excluded from these two isoform types. (This schematic figure is adapted from Shen et al. (2013))

Several combinations of both EC1 and IC3 variants allow for differential functioning within various tissue types. Both the PAC1-null and PAC1-hop1 receptors can stimulate AC in addition to PLC, but with differential levels of activation, which are dependent on type of ligand, tissue, and other splice events (Blechman and Levkowitz 2013). Conversely, the presence of the hip cassette significantly attenuates activation of the AC and PLC pathways (Spengler et al. 1993). PAC1-hop1, which is the major form expressed in sympathetic superior cervical ganglion neurons, is capable of also activating mitogen-activated protein kinase (MAPK) and Akt (a.k.a protein kinase B) signaling, promoting neuronal survival (May et al. 2010). Within the nervous system, the expression of specific isoforms has been observed at different times during neurogenesis, suggesting a distinct role for each PAC1 variant during brain development (Shneider et al. 2010). PAC1s, for example, is the more highly expressed isoform in human fetal versus adult brain tissue and thus may play a prominent role in development of the nervous system (Lutz et al. 2006). Interestingly, expression differences by gender have also been observed such that the expression of PAC1 is higher among females versus males in the hippocampus during prenatal development (Shneider et al. 2010).

2.3 PAC1 Is Localized to Neurons Within Several Regions of the Brain

Much of what has been gleaned regarding the localization of PAC1 has been determined in rat tissue. These studies have examined the expression of PAC1 in both the central nervous system and peripheral organ systems. PAC1 is detected in several tissue types including the eye, adrenal gland, pancreas, liver, lung, colon, lymphoid tissue (macrophages), and arteries (Lam et al. 1990; Hashimoto et al. 1996; Shioda et al. 1997; Pozo et al. 1997; D'Agata and Cavallaro 1998; Filipsson et al. 1998; Ekblad 1999; Huang et al. 2005). The prevalence of PAC1 in the central nervous system has also been examined extensively through detection of both mRNA transcript and PACAP binding sites in the rat brain. PAC1 is pervasive and expressed throughout several brain regions including the olfactory bulb, cerebral cortex, basal ganglia, amygdala, hippocampus, thalamus, hypothalamus, cerebellum, brainstem, and spinal cord (motor neurons) (Cauvin et al. 1991; Masuo et al. 1992; Hashimoto et al. 1996; Shioda et al. 1997). Although specific brain regions and cell types express different PAC1 isoforms, the major splice variant expressed in rat brain is the PAC1 short form that lacks either the hip or hop cassettes (referred to as the null isoform in humans) (Zhou et al. 2000). This isoform binds PACAP38 and PACAP27 with high affinity and preferentially activates adenylate cyclase through coupling with G_s-type G protein. Examination of subcellular localization by immunohistochemistry reveals dense staining of PAC1 within postsynaptic membranes and at both axon and dendritic synapses (Shioda et al. 1997). PAC1 has also been examined in embryonic cultures and is expressed in all embryonic stages suggesting a role in neuronal differentiation (Hirose et al. 2005).

Fewer studies report the localization of PAC1 in human tissues due to limited availability of experimental tissue. However, in order to better understand the function and prevalence of PAC1 in human tumors, comparative regions within healthy tissue have also been utilized to examine PAC1 expression patterns. In one study, VIP/PACAP receptors were found ubiquitously in most human epithelial tissues, particularly in the adrenal medulla, and epithelium of the uterus and pancreas (Reubi 2000). Another study reveals the expression of PAC1 in neurons of the small intestines/enteric nervous system (Schulz et al. 2004). The investigation of PACAP binding sites by receptor autoradiography revealed a strongly positive mRNA signal for PACAP within hypothalamic neurons (Oka et al. 1998). Despite only a few publications regarding the localization of PAC1 in the human brain, other researches investigating the role of PACAP/PAC1 in brain development provide clear evidence that PAC1 plays a prevalent role in neurological processes involving several regions on the brain (Lee and Seo 2014). Notably, these studies demonstrate a role for PAC1 in neuronal differentiation and repair (neurodegenerative disease).

2.4 PAC1 Is Involved in the Regulation of Stress

Several bodies of research have examined the impact of PACAP/PAC1 on stress pathways (Hammack and May 2014). In addition to its role in neurogenesis, PACAP is also a neurotransmitter responsible for the regulation and release of neuropeptide hormones including catecholamines and neuropeptide Y (NPY) (May and Braas 1995). The hypothalamic-pituitary-adrenal (HPA) axis, one of the main regulatory systems involved in stress response and secretion of corticotropin-releasing hormone (CRH) (from the hypothalamus) and adrenocorticotropic hormone (ACTH) (from the anterior pituitary), is tightly regulated in order to control the release of cortisol. PACAP contributes to this stress response system by activating the production of cortisol (in rodents, corticosterone) during prolonged stress (Stroth and Eiden 2010; Tsukiyama et al. 2011; Stroth et al. 2011). The release of cortisol by PACAP occurs through control of CRH secretion within the oval nucleus of the BNST (bed nucleus of the stria terminalis; BNSTov) and paraventricular nucleus (PVN) of the hypothalamus (Agarwal et al. 2005; Dore et al. 2013; Lezak et al. 2014; Roman et al. 2014) (Fig. 2.3). Induced stimulation of CRH through PACAP-/PAC1-mediated activation occurs through the cAMP/PKA signaling pathway which results in phosphorylation of the CREB transcription factor and its recruitment to the CRH promoter to initiate transcription (Agarwal et al. 2005).

Due to accumulating evidence, it has become widely recognized that PACAP plays an integral role in stress-related processes. However, as a neuropeptide ligand, PACAP must partner with one of its three G protein receptors in order to initiate its effect. Although PAC1 binds with highest affinity to PACAP, PACAP is not equally select in receptor binding and can stimulate biological systems through other receptor types (e.g., VPAC1 and VPAC2). For this reason, phenotypic outcomes related to an increase or decrease in PACAP cannot, deductively, be attributed to an interaction with PAC1 in all cases. Fortunately, several lines of evidence have come to light supporting a role of PAC1 in stress pathways, including the expression of PAC1 in the

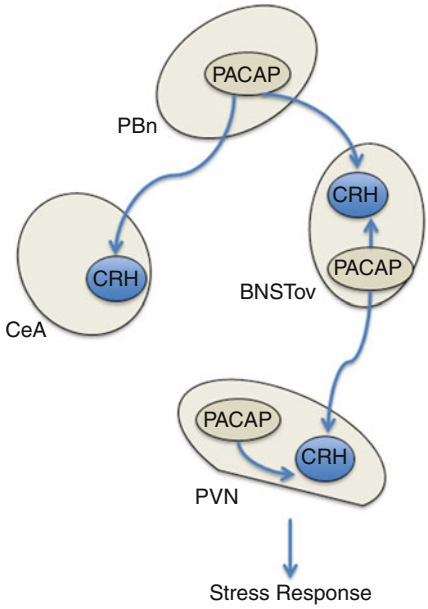

Fig. 2.3 Schematic diagram of the role of PACAP-PAC1 in the stress response. PACAP, localized to neurons within the parabrachial nucleus (PBn), oval nucleus of the BNST (BNSTov), and the paraventricular nucleus (PVN), can stimulate the release of corticotropin-releasing hormone (CRH) in various regions of the brain including the central nucleus of the amygdala (CeA). Signaling that results in the release of CRH in the BNSTov and PVN is involved in response to stress. (This schematic figure is adapted from Hammack and May (2014))

hypothalamus – a region of the brain involved in top-down hormone regulation and response to stress (Hashimoto et al. 1996; Shioda et al. 1997). PAC1 also localizes to the bed nucleus of the stria terminalis (BNST, increasingly appreciated for its role in unpredictable stress and anxiety-like behavior). Evident by the release of peripheral corticosterone when stimulated in rodent models, the BNST is also a region involved in the regulation of the HPA axis. Victor May and colleagues have shown an increase in PAC1 transcript in the BNST in response to chronic stress and that injection of PACAP into the BNST induces anxiety-like behavior (in rats) (Hammack et al. 2009). More recently, Roman et al. provide further evidence of PACAP signaling through PAC1 by showing that infusion of PAC1 agonist PACAP (6–38) can block the release of corticosterone and attenuate stress-induced behavior (Roman et al. 2014). Consistent with the later finding, one study that examined behavior for PAC1 (–/–) mice compared to wild-type littermates reports a statistically significant reduction in anxiety among the PAC1-null animals (Otto et al. 2001b).

2.5 Reduced Expression of PAC1 Results in Fear Learning Deficits

Phenotypes among mice with PAC1 and PACAP knockout or overexpression have been evaluated. Responses observed in PAC1-knockout mice, specifically, provide evidence that PAC1 is also involved in emotional behavior through mechanisms of fear learning. When compared to littermates of matched age and sex, PAC1 (–/–)-knockout mice exhibit a statistically significant level of reduced anxiety behavior in all of the three tests: open field, elevated zero maze, and elevated plus maze (Otto

et al. 2001b). The same research group identified a deficit in long-term potentiation within mossy fiber terminals of the hippocampus among the PAC1 (–/–)-null mice (Otto et al. 2001a). This finding leads to the investigation of associative learning behavior among the PAC1 mutant animals, revealing an impairment in contextual fear learning. Fear memory dysfunction in PAC1 (–/–) mice was corroborated by a second research group (Takuma et al. 2014). Additionally, microarray analysis of transcripts induced by the enhanced expression of the PAC1 short hop1 isoform within primary sympathetic neuronal cultures revealed an increase in brain-derived neurotrophic factor (BDNF) production (threefold) following acute treatment with PACAP27 (Braas et al. 2007). Similarly, PAC1 (–/–) mice have reduced levels of BDNF (Zink et al. 2004). Thus, it appears that PACAP/PAC1 signaling is positively correlated with BDNF expression. The significance of these findings is that BDNF, through BDNF/TrkB signaling, is integral to the formation and consolidation of fear memories (Yamada and Nabeshima 2003). Thus, reduced expression of BDNF as a consequence of PAC1 deficiency will result in the dysregulation of fear memory consolidation. These data provide yet more evidence for a pronounced role of PAC1 in fear learning. Interestingly, but perhaps not all that surprising, BDNF has also been implicated in posttraumatic stress disorder (PTSD), a neuropsychiatric condition accompanied by a dysregulation of fear memory consolidation (Andero and Ressler 2012).

Although most of the research evaluating the impact of PAC1 knockout on behavior has focused on the deficiencies of fear learning, these findings do not negate the significance of PAC1 in other processes that trigger stress response, namely, the HPA axis. Given the physical relationship between PACAP and PAC1 in particular regions of the brain, understanding the effects of PACAP overexpression or knockout in mice can also provide evidence regarding the multifaceted role of PAC1 in emotional behavior. One would expect that a knockout of PACAP would have similar effect as a knockout of PAC1 given that the receptor would become inactive in the absence of its high-affinity ligand. Consequently, we would expect PACAP knockouts to show reduced stress responses or a reduction in neuronal peptide hormones involved in initiating the stress response. One study reports that PACAP (–/–) mice showed a significant reduction in serum cortisol levels and depressive-like behavior after being exposed to social defeat stress (Lehmann et al. 2013). Other studies report contradictory findings with PACAP nulls exhibiting an increase in behavior associated with depression (Hashimoto et al. 2009; Gaszner et al. 2012). Regardless of the directionality of the effect, which may be due to differences in testing, a consistency in the relationship between altered PACAP/PAC1 functioning and effect on depression/anxiety behavior persists throughout the literature.

2.6 Genetic Variants of PACAP and PAC1 Have Been Implicated in Neuropsychiatric Illness

Several genome-wide association studies (GWAS) or candidate gene analyses have been performed and reveal genomic variants that are associated with neuropsychiatric disease (Table 2.1). These findings and subsequent investigations are crucial to

Table 2.1 Summary of *ADCYAP1R1* genetic association papers to date

ADCYAP1R1 SNP	Phenotype	Exposure	Population	Reference
rs2302475	Schizophrenia	N/A	Japanese	Hashimoto et al. (2007)
rs2267735	PTSD symptom severity	Civilian trauma	African American	Ressler et al. (2011)
rs2267735	PTSD symptom severity	Childhood maltreatments	Mixed; Caucasian, African American	Uddin et al. (2013)
rs2267735	PTSD symptom severity	Civilian trauma	African American	Almli et al. (2013)
rs2267735	Dark-enhanced startle	Unknown	Children; African American	Jovanovic et al. (2013)
rs2267735	Emotional numbing	Earthquake	Chinese	Wang et al. (2013)
rs2267735	PTSD	Acute trauma/ER visit	Mixed; primarily African American	Rothbaum et al. (2014)

our understanding of disease manifestation and can offer clues to prevention and treatment. In a small-scale GWAS, we found that polymorphisms within *ADCYAP1R1* (PAC1) associated with posttraumatic stress disorder (PTSD) in a human study cohort. Although these associations were not at a genome-wide level of significance, converging evidence combining the human genetic data with mouse amygdala gene expression data following fear conditioning implicated this pathway.

It is often difficult to assess whether or not the variant, initially identified, results in a change in expression or protein production and is indeed responsible for disease outcome. For this reason, significant associations between polymorphisms within a gene and the outcome of interest often trigger an investigation of other intragenic single-nucleotide variants (SNPs) that may be functionally responsible for disease risk. After a tag-SNP approach to testing multiple SNPs within this gene, an intronic SNP, rs2267735, was found to associate with PTSD diagnosis and PTSD symptoms among females only (Ressler et al. 2011). Based on current research (as described above), the implications of this finding were biologically relevant and the first evidence of a role for PAC1, specifically, in human disease.

This finding initiated further investigation among several other study cohorts. In some but not all, the association was replicated. In the first follow-up research study, Chang et al. report that the original finding did not replicate among females in two independent study cohorts of European and African descent (Chang et al. 2012). Since this initial study, three other studies have since provided evidence of replication. In a Chinese study cohort of earthquake survivors, rs2267735 associated with a subscale of PTSD, emotional numbing, in females only (Wang et al. 2013). In a study cohort of females from Detroit, the *ADCYAP1R1* SNP is associated with PTSD symptom severity when accounting for exposure to childhood maltreatment and race (Uddin et al. 2013; Almli et al. 2013). In a third replication study, emergency room patients experiencing severe physical trauma were recruited and assessed for

PTSD at several time points beginning at the time of visit to the emergency room (ER). This team found that study participants with the rs2267735 "CC" genotype were more likely to be diagnosed with PTSD at week 12 than those without this risk genotype (Rothbaum et al. 2014). Intermediate phenotypes of PTSD including an increase in dark-enhanced startle and hippocampal/amygdala reactivity in response to threatening stimuli (evident by fMRI) are also associated with the "CC" risk genotype of rs2267735 (Jovanovic et al. 2013; Stevens et al. 2014).

Unfortunately, the ability to replicate initial findings can be challenging due to differences in population stratification or environmental exposures. In fact, given the significant impact of trauma exposure on the development of PTSD, it may not be accurate to assess the association between genetic variants and PTSD without appropriately accounting for gene by environment interactions (Almli et al. 2013, 2014). DNA methylation in response to environmental stressors can also influence the expression of *ADCYAP1R1* independent of the effect induced by the SNP variant. The possibility also remains that there are other variant(s) in linkage, or which interact epistatically with rs2267735, that can account for differences between study cohorts. An in-depth, molecular characterization of rs2267735 on transcriptional regulation of *ADCYAP1R1* will be beneficial to our understanding of the underlying mechanisms involving PAC1 and PTSD. Although it is clear that a relationship likely exists between *ADCYAP1R1* expression and PTSD, it is less clear how this particular identified SNP may impact risk.

2.7 Epigenetic Regulation Can Impact Disease Outcome

Epigenetic regulation of gene expression comes in several varieties. The more classically understood mechanism of epigenetic regulation is DNA methylation at cytosines (5-methylcytosine (5mC)) within CpG islands that overlap with gene promoters and typically result in transcriptional repression. Cytosine methylation is not limited to these regions of the genome and can occur within enhancers, insulators, and transcription factor-binding sites throughout the genome (Schübeler 2015). Methylation at these sites has differential effects on regulation of gene expression and can even have effects on distant genes within the same or different chromosomes (Aran et al. 2013). In fact, in the human brain, the majority of methylated CpGs are within intragenic and intergenic regions with only 3% of methylation represented at CpG islands (Maunakea et al. 2010). Patterns of DNA methylation and transcriptional regulation differ across cell type and tissue as a biological necessity for cell-type-specific identity and processes. Kozlenkov et al. examined the prevalence of CpG methylation within neurons in medial orbitofrontal cortex (mOFC) of the human prefrontal cortex, comparing methylation patterns in these cells to nonneuronal cells (mostly glial). In neurons, they identified a predominance of CpG methylation within predicted enhancers located distally from transcription start sites (Kozlenkov et al. 2014). Also, compared to nonneuronal cell types, they observed an excess of differential methylation near neuron-specific transcription factor-binding sites.

Given the variety of epigenetic modification types and the variability of epigenetic marks in specific regions of the genome, the possibilities for gene regulation through epigenetic mechanisms are extraordinary. Histone modifications are another type of epigenetic regulation within the genome that should not be overlooked. In fact, Bredy et al. provide evidence for active histone acetylation at the promoter of BDNF during fear conditioning or fear conditioning followed by fear extinction. In particular, acetylation of histone H4 promotes fear extinction by inducing increased expression of BDNF (Bredy et al. 2007). Adding to the diversity of epigenetic regulation, passive or active demethylation of 5mC can also be converted to an intermediate methyl state, 5-hydroxymethylcytosine (5hmC), through the action of enzymes known as ten-eleven translocation (TET) family of proteins (Tahiliani et al. 2009). 5hmC is thought to be a secondary DNA methylation mark with its own regulatory function. An enrichment of 5hmC has been identified in brain tissue, specifically within synaptic genes, playing a role in splicing of these genes (Khare et al. 2012). 5hmC modification has also been implicated in the regulation of transcription factors involved in neurodevelopment (Wang et al. 2012). Interestingly, the 5hmC intermediate state of demethylation has also been implicated in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS). It is not difficult to imagine that research evidence will soon implicate 5hmC in the development and/or prevention of neuropsychiatric disorders (Kato and Iwamoto 2014). Complete methylation can also occur when 5hmC is further oxidized to produce 5-formylcytosine and 5-carboxylcytosine during the process of active demethylation. This provides a potential "on-off" switch for genetic regulation.

Epigenetic changes in the genome allow for cell differentiation as well as cell-specific processes. However, these changes also occur in response to one's environment. Considerable evidence has been found, revealing epigenetic marks that result from childhood maltreatment or high stress during adolescence (McGowan et al. 2009; Klengel et al. 2013; Essex et al. 2013; Mehta et al. 2013). These findings show, without a doubt, that epigenetic alterations play a key role in the development of neuropsychiatric outcome. Klengel et al. provide a thorough review of epigenetic alterations, particularly DNA methylation, that result in stress-related psychiatric disorders, including major depressive disorder, PTSD, and suicide (Klengel et al. 2014).

2.8 Patterns of DNA Methylation in PACAP and PAC1 Result in Altered Expression and Disease

The first evidence of disease related to differential methylation of *ADCYAP1* (PACAP) was observed in cervical cancer (Jung et al. 2011). Though typically unmethylated in normal cells, the CpG island near the promoter of *ADCYAP1* was found to be hypermethylated in several cervical cancer cell lines. Furthermore, hypermethylation was observed in early stages of carcinogenesis. Evidence of DNA

methylation, in the CpG island of *ADCYAP1R1*, that results in a diseased state has been reported in at least two studies. Following the identification of the PTSD risk polymorphism (rs2267735), it was hypothesized that DNA methylation within *ADCYAP1R1*, which could affect PAC1 expression state, would correlate with PTSD outcome. Indeed, this was the case. Subjects with PTSD have higher levels of *ADCYAP1R1* methylation within a CpG site of the *ADCYAP1R1* CpG island (cg27076139) (Ressler et al. 2011). In a second study, DNA methylation near the promoter of *ADCYAP1R1* at CpG cg11218385 was found to associate with increased odds of developing asthma among children ages 9 and older that were exposed to violence (Chen et al. 2013).

PACAP and PAC1 are ubiquitous proteins subject to several types of regulation, and research continues to reveal new mechanisms for such control. As bisulfite sequencing of DNA allows for more in-depth characterization of CpG methylation patterns, it is anticipated that more evidence will come to light revealing a more pronounced role of DNA methylation in expression and subsequent disease state.

2.9 Regulation of PAC1 Can Occur Through Changes in Isoform Expression Produced by RNA-Binding Proteins

Natural occurring splice variants may be advantageous to cell survival under certain conditions or may add specificity to function in a cell-type-specific manner. It is possible to imagine that polymorphic allele variants can affect splicing mechanisms within the gene and generate conformational changes in key protein regions and/or impede the cell's ability to effectively regulate PACAP/PAC1 signaling pathways as necessary. Given the role of PACAP in stress pathways, neuropsychiatric disorders, and other disease states, it is feasible that such variants may become evident during investigation of disease association. Regulation of PAC1 splicing is also affected by neuronal-specific RNA-binding proteins, which themselves can be genetically or epigenetically modified (Blechman and Levkowitz 2013).

Evidence for stress-induced regulation of PAC1 splicing currently exists. A2BP1 is a UGCAUG sequence-specific RNA-binding protein that regulates splicing of the hop cassette (exon 15) in the *ADCYAP1R1* (PAC1) transcript (Amir-Zilberstein et al. 2012). In a novel experiment to assess potential changes in PAC1 splicing in response to stress, PAC1 isoforms were measured in the paraventricular nucleus (PVN) of mice exposed to stress-inducing stimuli (footshock or restraint). In response to the stressor, PAC1 null/normal (no N-terminal deletion or cassette insertion) stimulates the activation of CRH transcription. This response is later "turned off" during stress recovery by alternative splicing that results in an increase of the PAC1-hop isoform, terminating stress-induced CRH production. Alterations, including epigenetic changes, can interfere with the regulation of this RNA splicing mechanism, disrupt homeostasis, and result in the dysregulation of normal stress response. In fact, genetic variants of A2BP1 have been associated with bipolar disorder and autism (Le-Niculescu et al. 2009; Davis et al. 2012).

2.10 Regulation of PAC1 by Estradiol Is Another Epigenetic Mechanism Associated with Control of Expression

Several lines of evidence implicate estradiol in the increased expression of PAC1 (Apostolakis et al. 2005; Aenlle et al. 2009; Ressler et al. 2011). Our group has shown evidence of expression differences in PAC1 presumed to be a result of dysregulated transcriptional activation by estradiol (E2) and estrogen receptor alpha (Erα) at a putative estrogen response element (ERE) (Ressler et al. 2011). Preliminary evidence (*unpublished*) from our lab has also revealed a statistically significant increase in the expression of PAC1 within the BNST of mice exposed to both stress and E2 compared to mice exposed to stress only. Thus, it is possible that increased expression of PAC1, particularly as a result of repeat exposure to stress (Hammack et al. 2009; Hammack and May 2014), may be facilitated or enhanced by the presence of estradiol. Although a positive correlation between levels of estradiol and PAC1 expression has been exhibited, it remains unclear if this effect is due to direct binding of the E2-Erα complex to ERE(s). Regardless, epigenetic mechanisms that alter either genomic or non-genomic E2-Erα signaling pathways will likely impact E2-induced activation of PAC1 expression. One particular example is the epigenetic regulation of Erα by microRNAs (miRNAs) including miR-18a, miR-22, and miR-206 (Vrtačnik et al. 2014). DNA methylation, known to inhibit binding of transcription factors, may also impact binding of Erα to EREs (Boyes and Bird 1991).

2.11 Long Noncoding and MicroRNAs: Evidence for a Role of RNA in Epigenetic Regulation

Direct, genomic regulation of PACAP or PAC1 by noncoding RNAs has not yet appeared in the literature, although unpublished data (Zsolt Nyisztor et al.; 5th Biannual Conference of the Hungarian Neuroscience Society, 2015) revealed mir137 regulation of hip expression during retinal development. Of note, many SNP polymorphisms that associate with the disease are located within regions of the genome with unknown function. The significance of these findings is not readily clear. However, as we continue to learn more about genomic regulation, the functionality of these variants will shed light on novel regulation pathways, which likely involve noncoding RNAs such as miRNA, lincRNA, and lncRNAs. These RNAs have regulatory function and may likely impact the expression of genes involved in neuropsychiatric conditions. Currently, a SNP in a lincRNA that increases risk for PTSD has already been identified (Guffanti et al. 2013).

2.12 Future Directions

It is reasonable to consider that estradiol may play a key role in the regulation of PAC1 expression as a natural, adaptive response to stressful events, particularly given that PTSD and depression, resulting from maladaptive stress response, are

more prevalent among females who have natural fluctuations in estrogen hormone levels (Kessler et al. 1995; Zlotnick et al. 2001). In fact, Glover et al. demonstrate that the inability to extinguish fear, a biomarker of PTSD, is more prevalent among women with low estrogen compared to women with higher levels of estrogen (Glover et al. 2013). Further evidence from our group suggests that PTSD may be the result of dysregulated expression of *ADCYAP1R1* (PAC1), which under certain circumstances, particularly the presence of the "C" rs2267725 allele, cannot be appropriately regulated by the transcriptional enhancer activity of estrogen-bound estrogen receptor alpha (Erα) at an estrogen response element (ERE) (Ressler et al. 2011).

The intronic regions of *ADCYAP1R1* that contains the rs2267725 PTSD risk SNP and putative estrogen response element (ERE) are relatively conserved between humans and mice, particularly in the small stretches of nucleotide sequence with ERE consensus. It is difficult to utilize the mouse model to investigate the functional impact of individual, human SNP polymorphisms associated with the disease; however, interrogation of ERE/ER binding can certainly be assessed. This is of particular importance because it also allows for functional analysis using brain tissue and regions of the brain associated with response to estrogen. It also provides an *in vivo* system, reducing the uncertainties associated with *in vitro* models. Utilization of the mouse model system in future experiments will provide invaluable translational approaches to better understand the epigenetic regulation of PAC1 and behavioral outcomes.

In summary, PACAP and its receptor PAC1 are critical modulators of the stress and fear pathways, and their dysregulation has been implicated in risk for PTSD and a number of other stress-related disorders. Genetic variants, epigenetic alterations, and hormone regulation have been attributed to changes in PAC1 expression presumed to be responsible for dysregulated emotional behaviors and other disorders. By further focusing on the cellular function, localization, and expression of the PAC1 receptor, ongoing work will further elucidate the effects of altered expression of *ADCYAP1R1* on phenotypic outcomes, particularly neuropsychiatric disorders.

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Potential Conflict of Interest Dr. Ressler is a founding member of Extinction Pharmaceuticals which exists to develop D-cycloserine for use to augment the effectiveness of psychotherapy. He has patents pending for the use of D-cycloserine and psychotherapy, targeting PACAP for extinction, targeting tachykinin 2 for prevention of fear, and targeting angiotensin to improve extinction of fear. He has received no equity or income from any of these relationships within the last 3 years. He has received funding from NIH, HHMI, NARSAD, and the Burroughs Wellcome Foundation.

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Chapter 3
Prodynorphin Epialleles

Igor Bazov and Georgy Bakalkin

Abstract Dynorphins, the endogenous ligands for kappa-opioid receptors play an essential role in neuroendocrine regulation, stress response, reward processing, and mood control. These neuropeptides induce strong dysphoric and aversive effects. Polymorphisms in the prodynorphin (*PDYN*), the dynorphin-encoding gene, are associated with substance addiction and negative craving, while dynorphin mutations cause neurodegeneration in the human brain. Similarly to other neuropeptide genes, *PDYN* is expressed in selective neural circuits at extremely low tissue levels. A sophisticated epigenetic/transcriptional regulation by cell lineage-specific transcription factors (TFs), insulators, and silencers such as CCCTC-binding factor (CTCF) and RE1-silencing transcription factor (REST) along with mechanisms that control neuronal activity-dependent transcription may define spatial, temporal, and adaptive *PDYN* expression patterns. Impairment of the epigenetic control of *PDYN* expression may contribute to human pathological conditions including substance dependence, depression, and chronic pain. Epigenetic and environmental factors may mechanistically converge on the *PDYN* CpG-SNPs associated with a risk for alcohol dependence, and the resulting methylation signals may be translated into disease predisposition via alterations in *PDYN* transcription. Understanding the mechanisms that regulate neuropeptide epigenome and transcriptome is essential for understanding of neuropeptide-mediated functional connectivity within neural circuits which activities define cognition and behavior.

Keywords Neuropeptides • Prodynorphin • Transcription • Epigenetics • DNA methylation • Human brain

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3.1 Introduction

The fluctuating patterns of electrical and chemical activities in brain neural circuits define cognition and behavior. Functional connectivity within neural circuits is shaped by neuromodulators that modify neuronal excitability and synaptic efficiency. Neuropeptides are the largest family of neuromodulators that includes more than 300 peptides (Hokfelt et al. 2000; Nathoo et al. 2001; Bargmann 2012; Bargmann and Marder 2013; Civelli 2012; Marder 2012; Nassel 2009; Stoop 2012; Taghert and Nitabach 2012; van den Pol 2012); (see also http://www.neuropeptides.nl/). Neuropeptides exert specific, coherent effects on formation and rewiring of brain neural circuits and consequently on behavior. Dysregulation of neuropeptides may lead to neurological and psychiatric disorders including depression, schizophrenia, and substance addiction. Thus, destruction of tiny populations of orexin/hypocretin-expressing neurons and mutations in dynorphin opioid peptides cause narcolepsy and spinocerebellar ataxia (Li et al. 2014; Muschamp et al. 2014; Bakalkin et al. 2010).

Neuropeptides are synthesized in small neuronal subpopulations in the CNS and after release diffuse through volume transmission to their cognate receptors on proximally or distantly located cells. Several properties enable neuropeptides to regulate functional connectivity in neural circuits. First, a relatively long extracellular half-life allows neuropeptides to diffuse to and activate distant receptor targets (Ludwig and Leng 2006). In a large number of CNS loci, a particular neuropeptide and its receptors are localized in different brain regions (Herkenham 1987). The neuropeptide-receptor mismatch suggests that neuropeptides released into the extracellular space may create the dynamic "neuropeptide regulatory cloud" covering local neural circuits. Second, neuropeptides activate their cognate G-protein-coupled receptors at low, nanomolar concentrations that allow them to function at substantial distance from their release sites and produce coherent effects on both proximal and distant neurons in a circuit. Third, through activation of various intracellular signaling cascades, neuropeptides exert a great variety of cellular effects including changes in membrane excitability, gene transcription, and synaptic and morphological plasticity (Pradhan et al. 2012; Theodosis et al. 1986; Bruchas and Chavkin 2010; Martin-Kleiner et al. 2006; Przewlocki 2004; Seybold 2009). Fourth, neuropeptides are capable to rewire or reprogram their target circuits by inducing priming effects (Ludwig et al. 2002, 2005) and tolerance (Bodnar 2013; Christie 2008; Harrison et al. 1998). The priming is the ability to prime vesicle stores for activity-dependent release, which might underlie changes in behavior. In the area reachable by slowly diffusing neuropeptide, most cells would be affected for a long time, while persistent activation may be reduced through receptor desensitization or internalization resulting in tolerance in cellular response.

Most neurons express neuropeptides, and multiple neuropeptides may be expressed by a single neuron. A neuropeptide phenotype is likely the most variable neuronal characteristic (Hokfelt et al. 1986, 1990; Skofitsch et al. 1985; Zupanc 1996). As a rule, each neuropeptide is expressed in tiny neuronal subpopulations

characterized by the CNS- and CNS area-specific patterns. Identification of transcriptional and epigenetic mechanisms that define neuronal subtype- and circuit-specific neuropeptide expression is a cornerstone in understanding of formation and functions of neural circuits.

The opioid peptide precursors are encoded by four genes: prodynorphin (*PDYN*), proenkephalin (*PENK*), proopiomelanocortin (*POMC*), and pronociceptin (*PNOC*) that give rise to dynorphins, enkephalins, endorphins, and nociceptin, the endogenous ligands for opioid receptors. These peptides act through G-protein-coupled κ-, δ-, μ-, and nociceptin receptors (KOR, DOR, MOR, and NOR). Dynorphins are extraordinarily potent peptides (hence the name) that function as endogenous ligands for the KOR (Chavkin et al. 1982; Smith and Lee 1988; Zhang et al. 1998). Dynorphins are released from nerve terminals to cause presynaptic inhibition and also from dendrites to cause retrograde inhibition of excitatory afferents. KOR activation is acutely inhibitory and results in stimulation of mitogen-activated protein kinase (MAPK) pathways (ERK1/2, p38 MAPK, and c-Jun kinase) (Bruchas and Chavkin 2010). Dynorphins are characterized by distinct anatomical expression patterns in the brain with higher expression in the striatum, hippocampus, hypothalamus, and amygdala (Fallon and Leslie 1986; Hurd 1996; Nikoshkov et al. 2008; Merchenthaler et al. 1997; Morris et al. 1986). The dynorphin/KOR system plays a role in a variety of physiological processes including neuroendocrine regulation, nociception, motor control, cardiovascular function, respiration, temperature regulation, feeding behavior, and stress response as well as in reward processing and mood control (Aldrich and McLaughlin 2009; Holden et al. 2005; Knoll and Carlezon 2010; Mika et al. 2011; Przewlocki and Przewlocka 2001; Nogueiras et al. 2012; Chavkin 2013; Schwarzer 2009; Bruijnzeel 2009). The most distinct feature of KOR agonists is that they are profoundly dysphoric when administered to humans (Pfeiffer et al. 1986) and aversive when given to rodents (Shippenberg et al. 2001, 2007; Shippenberg and Herz 1986; Wee and Koob 2010; Todtenkopf et al. 2004; Land et al. 2008). Dynorphins are released during exposure of rats or mice to stressful behavioral experiences (Mague et al. 2003; McLaughlin et al. 2003). Stress-induced dysphoria or anxiety is known to increase the risk of drug abuse in people (de Kloet et al. 2005) and to reinstate extinguished drug seeking in rodents (Shaham et al. 2000). Repeated stress exposure produces dynorphin-dependent dysphoria (Bruchas and Chavkin 2010; Bruchas et al. 2010; Knoll and Carlezon 2010). Pharmacological and genetic manipulations with the opioid receptors alter alcohol consumption in animals (Altshuler et al. 1980; Cichelli and Lewis 2002; Davidson and Amit 1997; Hall et al. 2001; Logrip et al. 2009; Myers et al. 1986; Shippenberg et al. 2007; Walker et al. 2011; Wee and Koob 2010). Animals lacking a functional PDYN/KOR system through either genetic deletion or receptor antagonism seem to be stress-resilient and less likely to seek drugs. These results suggest that the KOR-mediated dynorphin actions encode the dysphoric, aversive, and anxiogenic effects of stress in ways that increase the risk of drug abuse and addiction. The abstinent state during drug withdrawal is also profoundly dysphoric, and KOR activation by dynorphins may contribute to the intense craving that results in drug-seeking behaviors in humans and animal models of substance addiction. Consistently,

polymorphisms in the *PDYN* and KOR-encoding gene (*OPRK1*) are associated with alcoholism (Bart et al. 2005; Edenberg et al. 2008; Oslin et al. 2003; Ramchandani et al. 2011; Xuei et al. 2006, 2007), while *PDYN* variations are linked to negative craving in alcohol-dependent individuals (Karpyak et al. 2013), which is the desire for drinking in the context of tension, discomfort, or unpleasant emotions. Altogether these findings suggest that KOR antagonists may be therapeutically effective in treating depression and alcohol use disorders including negative craving and relapse prevention (Chavkin 2011).

In this review, we focus on epigenetic and transcriptional regulation of the *PDYN* gene understanding of which would shed light on general principles of the neuronal subtype- and neural circuit-specific expression of neuropeptide genes. Such selective regulation may be critical for shaping of functional connectivity by neuropeptides within and between neural circuits that ultimately define cognition and behavior.

3.2 Human *Prodynorphin* Transcripts

The *PDYN* gene produces several RNA variants coding for the full-length (FL) and several N-terminally truncated proteins in the human brain (Fig. 3.1). The previous study (Nikoshkov et al. 2005) and recent analyses by FANTOM consortium (Lizio et al. 2015) identified two main clusters of *PDYN* transcription start sites (TSSs) in *PDYN*. In the first cluster, two TSS groups determine the 5′-end of exon 1 (Fig. 3.1b). The second cluster is located in exon 4 of the gene. Transcription is initiated from the exon 4 TSSs in the brain regions characterized by high levels of expression including N. accumbens, caudate, putamen, hippocampus, and amygdala (Fig. 3.1c).

A canonical form of *PDYN* mRNA encoding the full-length protein (FL1-*PDYN* mRNA) consists of 4 exons and 3 introns (Fig. 3.1a). The second FL mRNA isoform (FL2) and transcripts with testis-specific alternative first exons (Taf I and Taf II) differ from FL1 in exon 1 variants (Nikoshkov et al. 2005; Telkov et al. 1998). Several 5′-truncated transcripts including alternatively spliced Sp1 and Sp2 and T1-T3 mRNAs produce N-terminally truncated (T) proteins lacking a signal peptide (Fig. 3.1d). Sp1 lacks a part of coding exon 4 and Sp2 exons 2 and 3 and a part of coding exon 4 (Nikoshkov et al. 2005) (Fig. 3.1d). TSSs of the T1-T3 mRNAs are located upstream or between the dynorphin-encoding sequences. TSSs scattered over exons were described for other genes (Carninci et al. 2006). In contrast to human, only one or two *Pdyn* transcripts, which code for the FL-proteins, have been identified in the rodent, guinea pig, lungfish, and amphibian (Dores et al. 2000; Pattee et al. 2003; Sharifi et al. 1999; Yuferov et al. 2001).

The FL1-*PDYN* mRNA showed a classic *PDYN* mRNA expression pattern, predominantly in limbic-related structures such as the ventral striatum, dorsal striatum patch compartment, accessory basal and cortical amygdala, dentate gyrus of the hippocampus, and entorhinal cortex. In contrast, FL2-mRNA shows more limited distribution with predominant expression over FL1 variant in the claustrum and

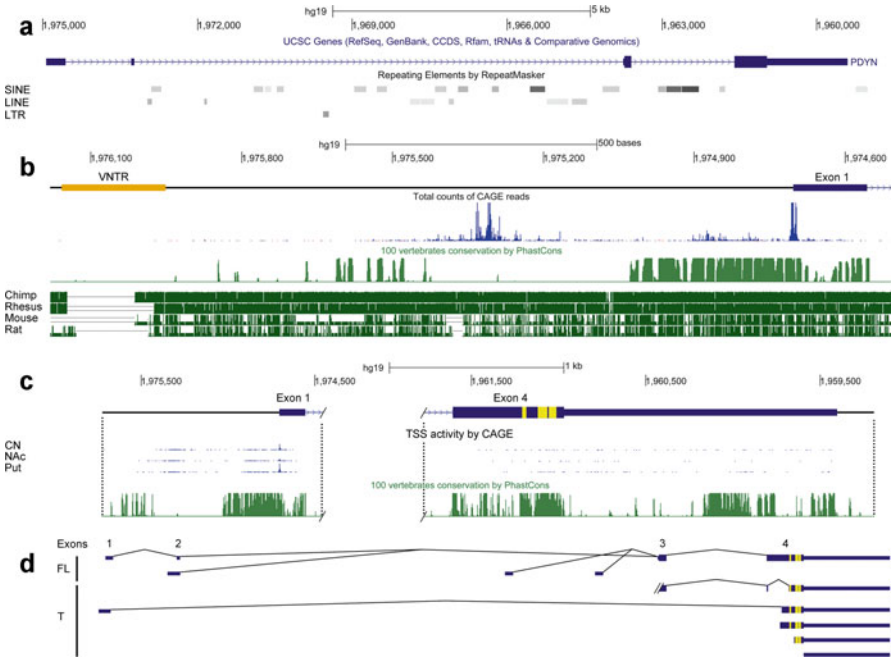

Fig. 3.1 Structure of human *PDYN* gene. (**a**) Genomic organization and repeating elements in human *PDYN* gene on chromosome 20. A screenshot from the UCSC Genome Browser on Human 2009 (GRCh37/hg19) Assembly. (**b**) Promoter region of human *PDYN* gene showing location of VNTR, two main clusters of TSS peaks, and degree of evolutionary conservation across 100 vertebrates and several selected species (UCSC Genome Browser). (**c**) Transcriptional activity of *PDYN* promoter region and coding exon 4 in highly expressing brain areas along with degree of evolutionary conservation across 100 vertebrates (UCSC Genome Browser). Noncoding, thin line in *dark blue*; coding, thick line in *dark blue*; dynorphin peptide-encoding sequences, *yellow*. *CN* caudate nucleus, *NAc* nucleus accumbens, *Put* putamen. (**d**) Known *PDYN* fetal and adult brain and testis-specific mRNA isoforms are shown. *FL* full length, *T* truncated. Exon numbers are shown for FL1-*PDYN*. Color key as in **c**

supraoptic hypothalamus. Even when present in the same brain structure (e.g., basal nucleus of Meynert and hypothalamus), the two *PDYN* mRNA forms were differentially expressed in discrete subnuclei (Nikoshkov et al. 2005).

Human *PDYN* exon 4, which encodes neuropeptides, has a promoter activity that may contribute to the intragenic initiation of transcription of T1 and T2 isoforms translated into two N-terminally truncated PDYNs with molecular size of 12 and 6 kDa, respectively. T1 and T2 proteins lack a signal peptide and are located in the cell nucleus and cytosol, respectively, suggesting their non-opioid functions (Nikoshkov et al. 2005).

A significant portion of the transcriptome, noncoding RNAs (ncRNAs), is involved in the regulation of the expression of neighboring protein-coding genes possibly through epigenetic mechanisms (Mattick 2001; Rinn et al. 2007). Long ncRNAs (lncRNAs) are the most abundant but not well-characterized class of

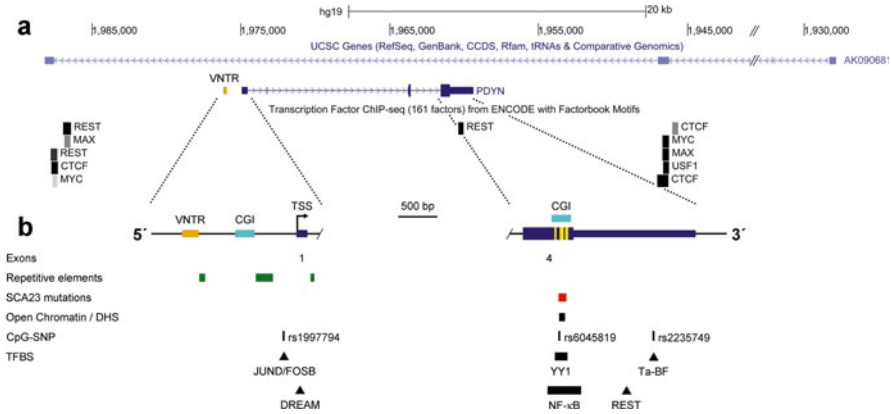

Fig. 3.2 Structure of human *PDYN* locus and regulatory elements. (**a**) Genomic organization of *PDYN* locus showing the location of *PDYN* gene within the antisense long noncoding gene *AK090681* and binding sites for selected TFs according to UCSC Genome Browser. (**b**) Regulatory elements within *PDYN* gene. Exon numbers are shown for FL1-*PDYN*. Localization of *PDYN* promoter VNTR, CpG island (CGI), repetitive elements, eight *PDYN* pathogenic mutations causing neurodegenerative disorder SCA 23 within short segment that include dynorphin A and B segment, and DNase I hypersensitivity peak overlapping with dynorphin A segment are shown. Three CpG-SNPs which show high association with alcohol dependence are indicated by *narrow vertical line* and SNP number. Putative binding sites for JUND/FOSB, DREAM, NF-κB, YY1, REST, and Ta-BF are shown by *arrowheads* and *black boxes*. Noncoding, *thin line* in *dark blue*; coding, *thick line* in *dark blue*; dynorphin peptide-encoding sequences, *yellow*

ncRNAs. The common strategy for analysis of lncRNAs is to study their genomic localization in relation to protein-coding genes. lncRNAs are derived from intergenic regions (long intergenic/intervening ncRNAs), overlap with main transcripts, and are also transcribed from the antisense strand of protein-coding genes. Expression of a protein-coding mRNA might be regulated by an antisense lncRNA derived from the same genomic locus. lncRNAs are highly versatile with a spectrum of novel activities that include recruiting nonselective transcriptional and epigenetic regulators to specific loci in the genome, forming nuclear bodies, modulating nuclear-cytoplasmic transport, and controlling local protein synthesis at synapses. Analysis of the human *PDYN* locus identified the *AK090681* gene encoding a 60,272 nucleotides long ncRNAs with 4 noncoding exons and 3 introns that are transcribed from the opposite strand of *PDYN* (Ota et al. 2004) (Fig. 3.2a) and may impact the *PDYN* expression. Our preliminary analysis identified robust and opposite differences in expression of *AK090681* and *PDYN* between the nucleus accumbens and cerebellum, the two areas with strongly different *PDYN* expression. The levels of *AK090681* mRNA were 20-fold lower, while those of *PDYN* 1,000-fold higher in the nucleus accumbens compared to the cerebellum.

Thus, *PDYN* transcription in human brain demonstrates high plasticity manifested in a number of transcripts, variations in their levels, brain area-specific patterns, and synthesis of several protein products that function as neuropeptide precursors, or nuclear and cytoplasmic proteins. Most exons are strongly conserved

in the human and rodent genomes. Exons that are only included in minor alternative splice forms (as opposed to the constitutive or major transcript form) are frequently characterized by their recent creation. The expression of minor human *PDYN* mRNA forms that are either spliced (Sp1, Sp2) or contain new exons (FL2, Taf I, Taf II) and that are apparently absent in other species supports the notion of an association of alternative splicing in the human genome with recent evolutionary changes. The impairment of the complex *PDYN* regulatory mechanisms at the levels of gene transcription and translation may contribute to human pathological conditions including chronic pain, substance dependence, and depression.

3.3 *PDYN* Promoter Mapping and Identification of Transcription Factors: Early Studies

3.3.1 Rodent Pdyn

Characterization of sequence requirements for *Pdyn* transcription led to identification of multiple promoter regulatory elements (Douglass et al. 1989). A 210 base-pair (bp) DNA fragment containing 88 bp of 5′-untranslated region (5′-UTR), the major cap site, and 122 bp of 5′-flanking DNA exhibited properties of a rat *Pdyn* promoter element active in a variety of cell lines (Douglass et al. 1989). Analysis of the rat *Pdyn* promoter region revealed the presence of a functional AP-1 element constituted by the noncanonical TGACAAACA sequence, a target for Fos/Jun transactivation (Naranjo et al. 1991). Deletion analysis identified a 358 bp fragment as the hormone-responsive sequence (−1858 to −1500 bp) containing three cAMP-responsive elements DynCRE1, DynCRE2, and DynCRE3 (Kaynard et al. 1992). These elements clustered at positions −1543, −1627, and −1659 relative to the RNA cap site conforming to cAMP regulatory element motifs (CGTCA) which positively regulate both basal and protein kinase A (PKA)-induced transcription (Douglass et al. 1994). The DYNCRE3 site, an AP-1/CRE-like element TGCGTCA, permitted high levels of transcriptional activity and dramatically responded to stimulations via the PKA second messenger pathway in cell lines (Messersmith et al. 1994). c-Jun is apparently involved in transcriptional activation through the DYNCRE3 site (Messersmith et al. 1996). DynCRE1, DynCRE2, and DynCRE3 elements were shown to be required for full activation of the rat *Pdyn* promoter in response to dopamine in striatal neurons, while AP-1 element did not confer significant activation. D1 dopamine receptor stimulation resulted in phosphorylated CREB binding to the DynCREs, with no significant protein binding to the putative noncanonical AP-1 (Cole et al. 1995). Data on TFs implicated in *Pdyn* regulation is summarized in Table 3.1.

Animal studies proposed that the persistent activation of ΔFosB, the C terminus-truncated FosB protein that is the AP-1 constituent, may be a common pathway for addictive disorders and that *Pdyn* is one of the transcriptional targets for this factor

Table 3.1 Transcription factors and their binding sites implicated in prodynorphin regulation rat *Pdyn*

Binding site	Sequence	Transcription factor	Location[a]	Reference
AP-1	TGACAAACA	Fos/Jun	−248	Naranjo et al. (1991)
DynCRE1-3	CGTCA	AP-1/CREB	−1858/−1500	Kaynard et al. (1992) Douglass et al. (1994)
DynCRE3	TGCGTCA	c-Jun	−1543	Messersmith et al. (1994) Messersmith et al. (1996)
Human *PDYN*				
DRE	GAGTCAAGG	DREAM	+40	Carrion et al. (1999)
E-es	TATGGCGGTTTTCTC	YY1	exon 4	Bakalkin et al. (1995)
κB	GGGGGCTTCT	NF-κB	exon 4	Bakalkin et al. (1994, 1997)
Pur		Pur	−480/−340	Unpublished observation

E-es Enkephalin-encoding sequence

[a]bp relative to TSS

(Zachariou et al. 2006). However, no detailed transcriptional study confirms this hypothesis, and no study assessed yet whether CREB and AP-1 with ΔFosB as a constituent regulate transcription of the human *PDYN* gene.

Not much is known about transcriptional networks that determine neuropeptide phenotype of neurons. Analysis of the developing mouse dorsal spinal cord demonstrated that the peptidergic transmitter phenotype of inhibitory neurons is specified by transcription factors Ptf1a, Pax2, and Neurod1/2/6 (Brohl et al. 2008; Huang et al. 2008; Wildner et al. 2013). A bHLH factor Ptf1a acts as upstream regulator and initiates expression of several downstream transcription factors in the future inhibitory neurons. Ptf1a is required for the expression of nociceptin/orphanin FQ, enkephalin, and dynorphin. Pax2 is a downstream target of Ptf1a and controls subsets of transmitter phenotypes, including the expression of nociceptin/orphanin and dynorphin, but is dispensable for enkephalin expression (Huang et al. 2008). Besides Pax2, Neurod1/2/6 factors, which act downstream of Ptf1a, are essential for dynorphin expression in dorsal horn (Brohl et al. 2008). Bhlhb5, a transcription factor, is expressed in several neuronal subtypes during development, and its deletion resulted in a loss subset of inhibitory neurons (termed B5-I neurons) in dorsal spinal cord. B5-I neurons inhibit itch and correspond to specific neurochemically defined populations that produce dynorphin (Kardon et al. 2014). In mouse striatal development, a LIM-homeodomain transcription factor Islet-1 specifies the cell fate of striatonigral neurons by regulating their genetic profiles, affecting a set of striatonigral-enriched

Table 3.2 Transcription factors implicated in *PDYN* cell-specific transcription in mouse spinal cord and striatonigral system

	Transcription factor	Reference
Dorsal spinal cord	Ptf1a, Pax2, Lbx1	Huang et al. (2008)
	Ptf1a, Neurod1/2/6, Lhx1/5	Brohl et al. (2008)
	Bhlhb5	Kardon et al. (2014)
Striatonigral system	Islet-1	Lu et al. (2014a)

genes which includes *Pdyn*, and orchestrating survival, differentiation, and axonal projections (Lu et al. 2014a). Thus, a cell-lineage-specific dynorphin expression is controlled at least in part by Ptf1a, Pax2 Neurod1/2/6, and Bhlhb5 in the dorsal spinal cord and Islet-1 in the striatum. However, transcriptional mechanisms that mediate action of these TFs have not been yet identified. Data on TFs that determine the dynorphinergetic neuronal phenotype in mouse spinal cord and brain is summarized in Table 3.2.

3.3.2 Human PDYN

PDYN promoter region 1.25 kb upstream of main TSSs is not conserved across vertebrates, besides the 330 bp sequence near to TSSs (Fig. 3.1b). However, the conservation is strong between humans and great apes and to less extent between humans and monkeys, while it is weak between human and rodents (Fig. 3.1b). The promoter region may represent a unique human cis-regulatory trait that is consistent with hypothesis on the recent positive selection of opioid cis-regulation in humans (Rockman et al. 2005). Lack of the conservation makes rodent models unsuitable for analysis of human *PDYN* promoter function.

The presence of promoter activity in 1 kb fragment of human *PDYN* promoter (fragment B encompassing 975 bp upstream to 25 bp downstream of the exon 1/intron A boundary) and the existence of upstream negative regulatory element(s) residing within 695 bp fragment (fragment A) located further upstream were demonstrated (Telkov et al. 1998). Deletion analysis and site-directed mutagenesis of a 1.8 kb genomic fragment encompassing the promoter region (from +150 to −1660 bp) of the human *PDYN* gene identified a minimal inducible 300 bp promoter fragment (from −150 to +150 bp) responsible for basal and PKA-regulated transcription in cell lines (Carrion et al. 1998). Within this region, a downstream response element (DRE, GAGTCAAGG) centered at position +40 in the 5′-UTR functions as a transcriptional silencer. DRE is targeted by the Ca^{2+}-binding transcriptional regulator DREAM (Carrion et al. 1999). DREAM-deficient mice demonstrate increased *Pdyn* expression (Cheng et al. 2002). A mechanism for DREAM-mediated cyclic AMP-dependent derepression that operates independently of changes in nuclear Ca^{2+} was discovered; with a direct protein-protein interaction between DREAM and alphaCREM, the CREM repressor isoform

prevents binding of DREAM to the DRE (Ledo et al. 2000). Thus, Ca^{2+} signaling pathway engages DREAM as an effector to derepress *PDYN* transcription that occurs in a kinase-independent manner (Campos et al. 2003).

Several other TFs including Yin-Yang1 (YY1) and nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) have also been implicated in the regulation of *PDYN* gene (Bakalkin et al. 1994, 1995, 1997). Interestingly, target DNA elements for these TFs are located in the coding part of exon 4 where they overlap with the dynorphin-encoding sequences implying that short, repetitive, and conservative neuropeptide-encoding sequences may function as target elements for TFs. Indeed, an exon 4 *PDYN* fragment comprising these sequences demonstrates ability to activate a reporter gene in cellular experiments (Nikoshkov et al. 2005). Data on TFs implicated in *PDYN* regulation is summarized in Table 3.1.

Human *PDYN* promoter and intron A also contain several repetitive DNA elements with high degree of similarity (>0.7) to retrotransposons, including MIRb repeat element of Short Interspersed Nuclear Element (SINE) group, and HERVIP10FH and HERV-K14CI endogenous retrovirus (ERV) group flanked by long terminal repeats (LTR), identified using CENSOR software tool (http://www.girinst.org/censor/) (Fig. 3.2b). Retrotransposons can be exapted (a process in which a feature acquires a function that was not acquired through natural selection) in evolution providing a source of regulatory elements, and LTRs may function as alternative promoters (Feschotte and Gilbert 2012; Gogvadze and Buzdin 2009). Another example is *POMC*, an ACTH and endorphin opioid peptide precursor gene, in which neuron-specific enhancers represent a conserved CORE-SINE retrotransposon (Santangelo et al. 2007). The *PDYN* HERVIP10FH is capable of binding sequence-specific, single-stranded DNA-binding proteins of Pur family (unpublished observation). Pur-alpha and Pur-beta proteins bind to both single-stranded DNA and RNA and functions in DNA replication and transcription and mRNA translation. They are critical for brain development (White et al. 2009) and through binding to CGG repeats may suppress CGG-mediated neurodegeneration (Jin et al. 2007). A role of retroviral elements in regulation of *PDYN* transcription remains to be investigated. In conclusion, (i) compared to many other neuronal genes, transcriptional regulation of both rodent and human *PDYN* gene has not yet been well disentangled; and (ii) TFs and mechanisms of cell-type- and brain area-specific and neuronal activity-dependent transcription have not been identified.

3.4 Genetic Factors

Structural genetic variants including SNPs may affect phenotype via changes in gene expression. Molecular mechanisms mediating effects of regulatory polymorphisms on gene transcription have not yet been comprehensively characterized; however, existing evidence points to creation or disruption of binding sites for transcription factors (Knight 2005; Wray 2007).

Human genetic studies revealed several *PDYN* SNPs associated with memory, emotions, addiction, and alcoholism. Elderly humans who are carriers of the minor

alleles of *PDYN* promoter rs1997794 and 3′-UTR rs910080 demonstrate higher episodic memory scores than homozygote carriers of the major allele (Kolsch et al. 2009). Also, a haplotype including these two SNPs and intronic rs2235751 was associated with better episodic memory scores (Kolsch et al. 2009). Human volunteers bearing the T allele of rs1997794 showed reduced fear extinction and a significantly diminished functional connectivity between amygdala and ventromedial prefrontal cortex (Bilkei-Gorzo et al. 2012).

The strong association was evident between alcohol dependence and multiple SNPs in the 3′-UTR, exons 3 and 4, as well as SNPs at the 5′-promoter and upstream region (Xuei et al. 2006). A haplotype block of six SNPs associated with alcohol dependence was found in the 3′-UTR (Xuei et al. 2006). The risk haplotype CCT of 3′-UTR rs910080, rs910079, and rs2235749 was significantly associated with cocaine dependence and with combined cocaine dependence and cocaine/alcohol codependence in Caucasians and lower expression of *PDYN* in both the caudate and nucleus accumbens (Yuferov et al. 2009). A candidate haplotype containing the *PDYN* rs2281285-rs1997794 was associated with alcohol dependence and propensity to drink in negative emotional states (Karpyak et al. 2013). Genotype distributions of *PDYN* rs1997794 and rs1022563 in the promoter and 3′-UTR were found to be associated with the risk of opioid dependence in females (Clarke et al. 2009). Increased odds ratios were also observed for rs910080 and rs199774 in female opioid addicts in European Americans (EAs) (Clarke et al. 2012).

Variable number tandem repeat (VNTR) sequences have been associated with complex disease traits. A notable example is the insulin/IGF-2 (*INS-IGF2*) VNTR located upstream of TSS in the insulin gene and involved in transcriptional regulation. This VNTR is present only in primates suggesting its recent evolutionary origin (Kennedy et al. 1995). The human *PDYN* gene harbors VNTR with polymorphic site (rs35286281) located upstream of the TSS. The humans carry from one to five copies of this 68 bp VNTR element, while nonhuman primates bear a single copy and other animals none (Rockman et al. 2005). All human copies of the VNTR element carry five substitutions that differentiate them from the sequence inferred for the last common ancestor of humans and chimpanzees. The *PDYN* VNTR contains AP-1 (TRE)-like binding site and weakly influences the inducibility of the gene (Zimprich et al. 2000). TPA stimulation elevated the expression of reporter gene from the promoter with three or four repeats (inducible HH genotype) compared to one or two repeats (noninducible LL genotype) in cells (Rouault et al. 2011). Effects of the repeats were cell-type specific and dependent on their number. Consistently, in human striatum, a number of inducible repeats correlated with *PDYN* mRNA (Nikoshkov et al. 2008).

Associations of variants of this VNTR with temporal lobe epilepsy (Stogmann et al. 2002), protection against cocaine dependence or abuse (Chen et al. 2002), schizophrenia in Chinese population (Zhang et al. 2004), opioid dependence in the African-American population (Ray et al. 2005), methamphetamine dependence (Nomura et al. 2006), cocaine/alcohol codependence in African Americans (Williams et al. 2007), and heroin dependence (Wei et al. 2011; Saify et al. 2014) were reported. These findings should be considered with caution because replication

studies ended with negative results (Bovo et al. 2008; Dahl et al. 2005; Nikoshkov et al. 2008; Ventriglia et al. 2002). More recently, *PDYN* VNTR polymorphism was found to modulate monetary reward anticipation in the corticostriatal loop in fMRI study (Votinov et al. 2014). Individuals with HH genotype showed higher activation and stronger functional coupling than those with LL genotype in the medial orbitofrontal cortex (mOFC) when anticipating a possible monetary reward. Thus, individuals with the HH genotype have a larger sensitivity for upcoming rewards, resulting in a higher motivation to attain these rewards. This finding may explain differences between the genotypes with respect to addiction and drug abuse.

In expression studies, analysis of a single variation may be too simplistic and should be avoided in favor of designs that include most potentially functional polymorphic sites. Genes with complex expression profiles such as *PDYN* may harbor multiple functional genetic variants; their cis-regulatory regions may be more extensive and represent a larger mutational target, while their protein products may be involved in multiple biological processes. That increases the likelihood of balancing selection among diverse functional demands. Effects on transcription were assayed for 19 naturally occurring haplotypes of the *PDYN* 3 kb cis-regulatory region (Babbitt et al. 2010). The impact of these variants differed between cell types and across brain region, while the effects of some combinations of individual variants on expression level were not additive. At least six different variants affected transcript abundance in vitro. In the human cortical areas, rs1997794 was strongly associated with expression level (Babbitt et al. 2010), while VNTR polymorphism did not affect *PDYN* expression (Cirulli and Goldstein 2007). Consistently, in the human hippocampus, *PDYN* expression may also depend on the rs1997794 genotype (Taqi et al. 2011a). The T, low-risk allele of this SNP resides within noncanonical AP-1-binding element that may be targeted by JUND and FOSB proteins, the dominant AP-1 constituents in the human brain. The T to C transition abrogated AP-1 binding (Taqi et al. 2011a). Thus, the impact of genetic variations on *PDYN* expression is unexpectedly complex and context dependent, partially due to the cis genotype-cell interactions and epistatic interactions between nearby variants.

3.5 Epigenetic Mechanisms

3.5.1 PDYN *in Chromosomal Context*

In silico analysis of genome-wide data accumulated for a variety of cell lines identified several strong signals generated by TFs including CCCTC-binding factor (CTCF) in the *PDYN* locus (Fig. 3.2a). CTCF contains 11 zinc fingers, binds to a wide range of DNA sequences, and has diverse regulatory functions, including transcriptional activation/repression, insulation, and imprinting. CTCF may mediate intra- and interchromosomal contacts through the formation of loops between two CTCF-bound insulators (Hou et al. 2008). The formation of these loops could either

activate (Majumder et al. 2008) or repress gene transcription (Hou et al. 2008; Tanimoto et al. 2003) by facilitating or inhibiting interactions of the enhancer or inhibitor with promoter. Two strong peaks of CTCF bound to its response elements frame the *PDYN* gene in most cell lines (Fig. 3.2a). The *PDYN* CTCF peaks overlap with strong c-MYC, MAX, JUNB, JUND, cFOS, and YY1 signals. These peaks are located in exons 3 and 4 of the *AK090681* gene transcribed from the complementary strand (Fig. 3.2a). *PDYN* is also targeted by the REST transcription factor, in which two peaks are located ca. 12 kb upstream and in the 3′-UTR of the gene (Fig. 3.2a; see Sect. 3.5.5). The REST upstream peak is located close to the upstream CTCF binding element from the proximal to *PDYN* side. The *PDYN* transcription unit may be separated from *AK090681* and other genes in this area by CTCF-mediated chromatin looping, and interactions with these TFs may be critical for the locus-specific *PDYN* regulation (Fig. 3.2a).

PDYN transcription has not been extensively studied in chromosomal context; such analysis was hindered by the absence of appropriate cellular and animal models. Compared to the brain, human and rat cell lines express prodynorphin at much, 10–1,000-fold lower levels, and a residual *PDYN* expression in cell lines is not responsive to pharmacological treatments known to modify transcription of this gene in animal models (unpublished observations). As far as we are aware, no papers on the in vivo/ex vivo analysis of *PDYN* regulation in human brain besides the REST study, which is discussed under Sect. 3.5.5, have been published.

3.5.2 Epialleles of PDYN *SNPs Associated with Mental Disorders (the CpG-SNP Hypothesis)*

The HapMap database identified 2,252,113 C/T and G/A SNPs in the autosomal chromosomes. Of those, 34 % are located within a CpG dinucleotide and are named as CpG-SNPs (Sigurdsson et al. 2009). Sequences containing CpG dinucleotides are 6.7-fold more abundant at the polymorphic sites than expected (Tomso and Bell 2003). Such SNPs could serve as functional cis-regulatory variant by forming or disrupting a CpG, which methylation and hydroxymethylation are therefore allele specific, and could be linked to allele-specific gene expression. Genetic, epigenetic, and environmental factors have been hypothesized to influence the phenotype and the risk for the development of diseases through their effects on gene transcription. Mechanistically, these effects may be integrated through regulation of methylation of CpG-SNPs associated with a disorder, hence affecting expression of high- or low-risk allele (Fig. 3.3a). Thus, in addition to two SNP alleles, three epialleles formed by unmethylated, methylated, and hydroxymethylated cytosine in a CpG-SNP may exert diverse effects on the phenotype such as disease vulnerability by engaging different epigenetic mechanisms.

This hypothesis was addressed by analyzing methylation of *PDYN* CpG-SNPs associated with alcohol dependence in the postmortem human brain study

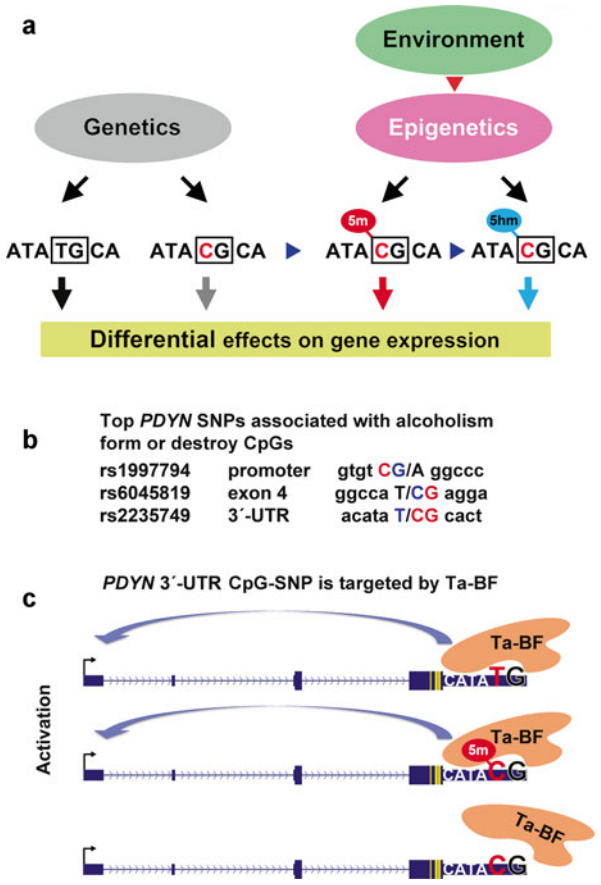

Fig. 3.3 The CpG-SNP hypothesis. (**a**) A hypothetical model showing how effects of the genetic, epigenetic, and environmental factors may (i) be mechanistically integrated through regulation of methylation and hydroxymethylation of CpG dinucleotides overlapping with SNPs and (ii) thereby influence the risk of a disease through effects on gene transcription. (**b**) Top *PDYN* SNPs associated with alcoholism with high significance are CpG-SNPs. Variants significantly associated with alcoholism are depicted in *blue* and nucleotides forming CpG sites are shown in *red* color. (**c**) Functional link between the methylation and gene expression could be mediated by T allele binding factor (Ta-BF) which binds to the T and methylated C alleles with higher affinity than unmethylated C allele of the 3′-UTR CpG-SNP. Binding of Ta-BF to the T or methylated C allele may result in activation of gene expression

(Taqi et al. 2011b). Analysis of *PDYN* genetic polymorphisms demonstrated that three (rs1997794, rs6045819, and rs2235749) out of five top *PDYN* SNPs associated with alcoholism with high significance are CpG-SNPs (Fig. 3.3b). The C allele of these CpG-SNPs was differently methylated in the human brain. In the dl-PFC of alcoholics, methylation level of the C, non-risk variant of 3′-UTR SNP (rs2235749; C>T) was increased. The *PDYN* 3′-UTR CpG-SNP methylation positively correlated with dynorphins suggesting a functional link between the methylation and gene expression. Furthermore, a T allele binding factor (Ta-BF), a 63 kDa protein

showing higher binding affinity for the T and methylated C alleles than for unmethylated C allele of the 3′-UTR CpG-SNP, has been identified in human brain (Fig. 3.3c). Due to the binding profile, this factor may regulate *PDYN* transcription through binding to the T allele or methylated C 3′-UTR CpG-SNP allele. *PDYN* expression and 3′-UTR CpG-SNP methylation may positively correlate if binding of Ta-BF to the methylated C allele results in transcriptional activation. Altogether, these findings suggest that the genetic, epigenetic, and environmental factors associated with a risk for alcohol dependence may mechanistically converge on the *PDYN* 3′-UTR CpG-SNP and that the resulting methylation signals may be translated into disease predisposition via alterations in *PDYN* transcription by such factors as Ta-BF. It would be essential to examine a role of this factor in *PDYN* regulation.

Integration of environmental, genetic, and epigenetic signals by their convergence on CpG-SNPs associated with a phenotype/disease may be a general phenomenon. This concept is supported by gene-specific and genome-wide human studies. Several best-known polymorphisms in neuronal genes associated with various mental phenotypes represent CpG-SNPs. These examples include CpG-SNPs of the catechol-O-methyltransferase (*COMT*), GABA(A) receptor beta(2) (*GABRB2*), and μ-opioid receptor (*OPRM1*) genes, in which methylation at these sites affects transcriptional efficiency and is linked with the phenotype (Oertel et al. 2012; Pun et al. 2011; Ursini et al. 2011; Mill and Petronis 2007; Sigurdsson et al. 2009; Hellman and Chess 2010; Xie et al. 2009). Analysis of type 2 diabetes demonstrated that 50 % of 40 SNPs associated with this disease represent a CpG-SNP that may function as DNA elements mediating development of the diabetic phenotype. These CpG-SNPs were differentially methylated, and the methylation level was associated with gene expression, alternative splicing, and hormone secretion in the human islets (Dayeh et al. 2013). A CpG site created by the G allele of a CpG-SNP of the growth differentiation factor 5 (*GDF5*) gene altered the binding affinity for SP1 and SP3 repressor proteins which have a higher affinity to the unmethylated allele, and this leads to an expression imbalance between both alleles (Reynard et al. 2014). A CpG-SNP in the promoter of cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*) gene is associated with oligoasthenoteratozoospermia and testosterone levels in infertile males, and the degree of methylation in the SNP site was high in colon and stomach tissue while low in the testis, kidney, and adrenal gland (Park et al. 2014). The tissue-specific DNA methylation pattern within the *CYP17A1* CpG-SNP was further associated with high *CYP17A1* expression in tissues with low methylation at the site. Intragenic CpG-SNPs can influence transcription elongation positively or negatively through alternative promoters or noncoding transcripts (Deaton and Bird 2011; Deaton et al. 2011; Maunakea et al. 2010). Methylation of a CpG-SNP can also play a role in the regulation of splicing by helping the splicing machinery to identify exons (Shukla et al. 2011) or by affecting recombination rates (Sigurdsson et al. 2009). Importantly, over 80 % of genetic variants at CpG sites (CpG-SNPs) are methylation-QTL loci, and CpG-SNPs account for over two thirds of the strongest methylation-QTL signals at the genome-wide scale (Zhi et al. 2013). As such, CpG-SNPs apparently have an essential role in normal and pathological processes linking genetic variation to epigenetic modifications induced by a changing environment.

3.5.3 *DNA Methylation*

Little is known about *PDYN* DNA methylation and its effects on gene transcription. *PDYN* methylation in human brain tissues and peripheral blood mononuclear cells (PBMC) was analyzed in a single study (Yuferov et al. 2011). *PDYN* has two short CpG islands that are located in the promoter and coding part of exon 4 (Fig. 3.2b). Methylation of the promoter CpG island differed substantially between brain tissues and PBMC and between two brain regions analyzed, the caudate and anterior cingulate cortex. One of the promoter CpG sites forming E-box DNA element, a target site for cMyc or USF1 transcription factors was methylated at low levels in both PBMC and brain tissues. The general conclusion was that *PDYN* DNA methylation profile was concluded to be typical for primary responsive genes with regulatory elements for both basal and tissue-specific transcription (Yuferov et al. 2011).

Gene body methylation generally received less attention than methylation of promoters. However, high cytosine methylation levels in exons (Nguyen et al. 2001) and a positive correlation between gene body methylation and gene expression levels were described for many genes (Ball et al. 2009; Jones 1999; Moen et al. 2013). Several transcription start sites, promoter activity, TF binding sites, and SNP associated with alcoholism were described for the *PDYN* CpG island located in exon 4 coding region (Bakalkin et al. 1994, 1995, 1997; Nikoshkov et al. 2005; Xuei et al. 2006). This island was found to be hypermethylated at similar levels in both brain tissues and PBMCs that strongly differ in *PDYN* expression (Yuferov et al. 2011). More detailed analysis of DNA methylation in this region demonstrated that methylation patterns were (i) conserved across human individuals, while (ii) strongly different between human tissues and cultivated human neuronal and nonneuronal cell lines, brain and peripheral tissues, and brain areas (preliminary data and (Bazov et al. 2011)). Several CpGs demonstrated features of variable methylation sites; their methylation was drastically (five to eightfold) different between brain areas and brain tissues and cell lines. The exon 4 methylation was abolished in DNMT1/3b-deficient cells and did not correlate with the *PDYN* FL mRNA levels. In this *PDYN* region, epigenetic mechanisms may regulate chromatin remodeling relevant for initiation of transcription within the exon 4 coding sequence, transcriptional elongation, and/or alternative splicing.

3.5.4 *Single-Stranded* PDYN *DNA Methylation and Formation of Noncanonical DNA Structures*

Nine missense mutations in the human *PDYN* gene that *cause* profound neurodegeneration in the cerebrum and cerebellum underlying the spinocerebellar ataxia type 23 (SCA23), a dominantly inherited neurodegenerative disorder, have been recently identified (Bakalkin et al. 2010; Jezierska et al. 2013; Saigoh et al. 2015). Generalized pathological changes including cerebral cortical and subcortical atrophy and agenesis of corpus callosum in patients carrying *PDYN* mutations

emphasize the fundamental role of dynorphins in regulation of neuronal functions and survival. These findings propose that the dynorphin-activated mechanisms may contribute to neurodegeneration and neuronal dysfunction in several neurological disorders including Alzheimer's disease, substance addiction, depression, and chronic pain, which all are characterized by dynorphin upregulation (Yakovleva et al. 2007; Bazov et al. 2013; Ossipov et al. 2007; Maximyuk et al. 2015).

Remarkably, eight out of nine *PDYN* SCA23 mutations are located in the 96 nucleotide dynorphin A and B sequence known as the big dynorphin domain (Big Dyn) (Fig. 3.2b). Big Dyn with dynorphin A as a core exerts strong non-opioid neurodegenerative activities that may underlie SCA23 pathogenesis (Maximyuk et al. 2015; Tan-No et al. 2001, 2002).

The localization pattern of SCA23 mutations in *PDYN* suggests that the Big Dyn-encoding domain is a mutational "hot spot." This domain also represents the short CpG island in which SCA23 mutations either eliminate or create a CpG site, thus suggesting a role of DNA methylation-/demethylation-dependent mutagenesis. Furthermore, this segment demonstrates a high DNase I hypersensitivity (Fig. 3.2b) that may be due to its presence in a partially denatured or a single-stranded form in the cell.

During gene transcription and DNA replication and recombination, helicases unwind double-stranded DNA to single-stranded DNA (ssDNA), which serves as a template for RNA and DNA polymerases (Patel et al. 2011). ssDNA has high conformational flexibility, allowing a variety of conformations and formation of noncanonical secondary structures such as cruciforms, G-quadruplexes, and triplexes. These structures may have regulatory roles in gene transcription (Sakamoto et al. 2001; Siddiqui-Jain et al. 2002), DNA replication (Pearson et al. 1996), and recombination (Faruqi et al. 2000; Napierala et al. 2002) and importantly are involved in mutagenesis (Lada et al. 2011; Sakamoto et al. 2001; Wells 2007).

Formation of noncanonical secondary DNA structure by the single-stranded Big Dyn-encoding *PDYN* fragment and the effects of CpG methylation on this process were assessed in vitro using native polyacrylamide gel electrophoresis (nPAGE), circular dichroism (CD) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy (Taqi et al. 2012). The presence of secondary DNA structures in the Big Dyn fragment was evident from differences in oligonucleotide mobility on nPAGE, from CD spectra, and from formation of A-T, G-C, and noncanonical G-T base pairs observed by NMR spectroscopy. Methylation at CpG sites prompted sequence-dependent formation of novel conformers, or shifted the equilibrium between existing ssDNA conformations (Fig. 3.4). The effects of methylation were sequence dependent and comparable in strength to the effects induced by point mutations in these DNA molecules (Taqi et al. 2012). The secondary structure calculations predict that energetically favorable Dyn A structures contain a loop-stem with two cytosines which methylation induces conformational shift in ssDNA fragment. The Dyn A loop-stem consists of bases 8–10 (CGC) paired with bases 25–27 (GCG) (Fig. 3.4b; see also Fig. 6 in Taqi et al. 2012). Similar free energies characterize pairing of the bases 8–10 (CGC) with 25–27 GCG and 28–30 GCG sequences. Both 26 and 29 cytosines, central in the stem, form CpG sites. Methylation of C26

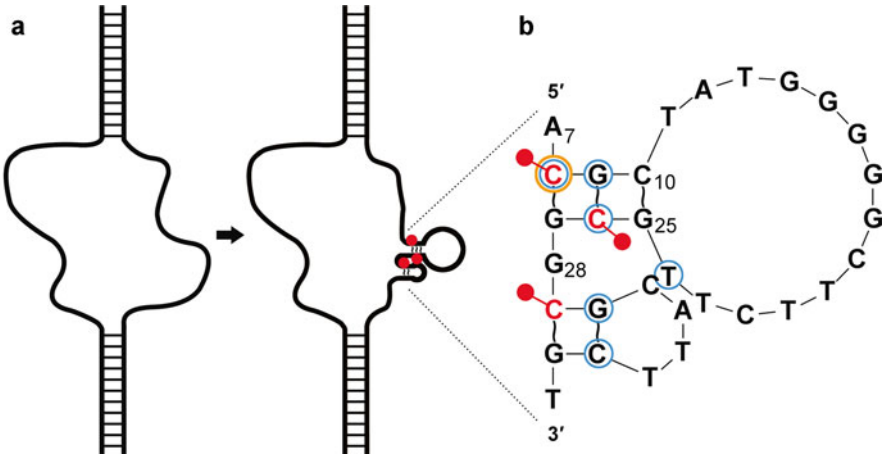

Fig. 3.4 Formation of noncanonical DNA structures by single-stranded DNA methylation. (**a**) Hypothetic model for the CpG methylation prompted sequence-dependent shift in ssDNA conformation. Cytosines which methylation affects ssDNA conformation are shown as *red circles*. (**b**) Energetically favorable Dyn A structure predicted by the secondary structure calculations represents a loop-stem with two cytosines which methylation induces conformational shift in ssDNA fragment. The Dyn A loop-stem consists of the bases 8–10 paired with bases 25–27. Similar free energies characterize pairing of the bases 8–10 with 25–27 and 28–30 sequences. Cytosines which methylation affects ssDNA conformation are shown in *red letters*. Pathogenic SCA23 mutations are shown in *blue circles*. Cytosine in *orange circle* is differentially methylated between human brain areas, peripheral tissues, and proliferating cells in culture. The presence of the differentially methylated CpG site and sequence-specific effects of CpG methylation on ssDNA conformation suggest a functional link between methylation/demethylation of this short sequence, its putative role in gene transcription or DNA replication, and generation of pathogenic mutations

and/or C29 may affect the preference of bases 8–10 to form pairs with the 25–27 or the 28–30 GCG triad. The actual secondary structures were not revealed in this study; however the spectroscopic and biochemical (nPAGE) data together with computational structure prediction strongly suggest that 5-methylcytosine and unmodified cytosine differentially influence the ssDNA secondary structure formation.

Noncanonical ssDNA structures may be formed by ssDNA under conditions of DNA supercoiling that affects gene regulation in pro- and eukaryotes (Hernandez et al. 2007; Krasilnikov et al. 1999; Wright et al. 2011). One example is the p53 tumor repressor protein which displays stronger binding to its DNA target site when the latter adopts a stem-loop conformation (Gohler et al. 2002). It is important to examine whether cytosine methylation may affect the conformational flexibility of ssDNA segments in vivo in a chromatin context under conditions of a crowded intranuclear environment and DNA superhelical stress. From biological standpoint, the conformational effects of methylation may be relevant for epigenetic regulatory events in a chromatin context, including DNA-protein or DNA-DNA recognition in the course of gene transcription and DNA replication and recombination when double-stranded DNA is unwinded to ssDNA.

3.5.5 PDYN *Is Targeted by REST in the Human Brain*

The RE1-*s*ilencing *t*ranscription factor (REST) (Chong et al. 1995) also known as *n*euron-*r*estrictive *s*ilencer *f*actor (NRSF) (Schoenherr and Anderson 1995) is a zinc finger protein that functions as a master regulator of neuronal phenotype. REST blocks transcription of its target genes by binding to 21 bp RE1 binding site/neuron-restrictive silencer element (RE1/NRSE). The cellular context critically defines the REST function(s). Equally important are the differences between neuronal and non-neuronal phenotypes and stages of cell differentiation. REST represses transcription in embryonic stem cells (ESCs) or nonneuronal cells. During neuronal differentiation and in neurons, REST protein level is decreased and loss of its binding from genomic sites is correlated with histone modifications such as increase in acetylation that activates transcription (Zheng et al. 2009). Action of REST is mediated through epigenetic mechanisms; upon binding to RE1 sites, REST recruits specific inhibitory enzymatic activities to target loci. REST is also an epigenetic modulator regulating medium- and long-term changes in gene expression. Often genes targeted by REST do not respond to physiological stimuli, and such dysregulation may contribute to transition to the pathological state (Bithell 2011).

REST regulatory elements were found in the tachykinin precursor 1 and tachykinin precursor 3 genes, arginine vasopressin gene (Coulson et al. 1999, 2000; Gillies et al. 2009; Li et al. 2008), and other neuropeptide genes (Lonnerberg et al. 1996; Mendelson et al. 1995; Carroll et al. 1995; Chin et al. 1994; Desmarais et al. 1992; Dong et al. 1995; Hoyle et al. 1994; Kraner et al. 1992; Li et al. 1993; Symes et al. 1992), suggesting that this TF functions as a general regulator of multiple neuropeptide pathways and neuropeptide neuronal phenotypes (Kuwabara et al. 2005; Lunyak and Rosenfeld 2005).

To identify TFs that regulate human *PDYN*, we screened chromatin immunoprecipitation sequencing data on 161 TFs from 91 cell lines generated by ENCODE (Gerstein et al. 2012; Wang et al. 2012). Besides CTCF, the most compelling evidence determined by signal intensity, motif score, and number of cell lines has been obtained for REST (Fig. 3.2a). *PDYN* locus contains two REST target sites, which were also identified in other studies (Bruce et al. 2004; Johnson et al. 2007). These sites are located ca. 12 kb upstream and in the 3′-UTR of the *PDYN* gene. They are canonical or nearly canonical and bound by REST in all or most cell lines analyzed (Rockowitz et al. 2014).

Whether REST may control *PDYN* transcription was assessed using a dominant negative mutant REST protein that contains the REST DNA-binding domain but no terminal repressor domains (Tapia-Ramirez et al. 1997). Displacement of REST from its *PDYN* binding sites resulted in decreased REST binding to both *PDYN* RE1s and increased *PDYN* transcription in cellular models. The in vitro data were complemented by ex vivo analysis of REST binding to *PDYN* in the human prefrontal cortex. ChIP-qPCR assay developed for analysis of frozen human brain tissues identified REST binding to the RE1 located upstream of *PDYN*, but not to the 3′-UTR RE1 element.

REST regulates and is regulated by the microRNA MIR-9, and together these molecules may mediate a switch in chromatin remodeling complexes that is essential for neural development (Packer et al. 2008; Yoo et al. 2009). The mechanism by which MIR-9 downregulates REST is unknown; one possibility is that this microRNA blocks translation of REST mRNA resulting in decreased REST protein levels. Taking advantage of this interaction, REST-mediated effects of MIR-9 on *PDYN* transcription were analyzed in cellular models (Henriksson et al. 2014). The MIR-9-induced REST downregulation resulted in decreased REST binding to both the upstream and 3′-UTR *PDYN* RE1 elements that leads to increased *PDYN* expression. Analysis of human brain identified strong negative correlation between REST and MIR-9 supporting the negative feedback hypothesis. Altogether these findings suggest that REST represses *PDYN* expression in the human brain, while the expression of this TF is under control of MIR-9 microRNA (Henriksson et al. 2014).

Altered *PDYN* and dynorphin peptide expression is observed in the brain of addicted subjects and patients with Alzheimer's disease and epilepsy (Yakovleva et al. 2007; Bazov et al. 2013; de Lanerolle et al. 1997). These disorders are also associated with altered MIR-9 and/or REST expression (Ooi and Wood 2007) that may have mechanistic link to *PDYN* transcription. Thus, in Alzheimer's disease characterized by high MIR-9 and low REST expression (Holohan et al. 2012; Lu et al. 2014b), the increased dynorphin peptide expression (Yakovleva et al. 2007) may result from MIR-9-mediated downregulation of REST. This mechanism may contribute to pathogenesis of Alzheimer's disease because the elevated pathogenic dynorphin (i) correlates with the neuritic plaque density and (ii) induces neurodegeneration in animal and human brain (Yakovleva et al. 2007).

3.6 Conclusions

The PDYN-derived opioid peptides are critical regulators of neural circuits processing emotions, sensory information, and reward (Koob and Le Moal 2005; Walker et al. 2012; Bruijnzeel 2009; Chavkin 2013; Schwarzer 2009). Dynorphin dysregulation may contribute to additive disorders, depression, pathological pain, and Alzheimer's disease (Bazov et al. 2013; Maximyuk et al. 2015; Ossipov et al. 2007; Yakovleva et al. 2007; Butelman et al. 2012; Shippenberg et al. 2007; Carlezon et al. 2009). However, it is not clear whether epigenetic and transcriptional mechanisms or more distal processes such as RNA translation and protein processing are impaired. *PDYN* epigenetic and transcriptional regulation in normal and pathological brain has not been addressed yet at the extent similar to that for other neuronal genes (e.g., *BDNF*, *POMC*, and *COMT*). *PDYN* analysis is hindered by the absence of appropriate cellular and animal models and its extremely low tissue expression levels. As the result, *PDYN* promoter/enhancer elements and their epigenetic modifications driving transcription have not yet been comprehensively characterized.

PDYN and other neuropeptide genes are special in their very restricted expression profiles and low tissue mRNA levels. A sophisticated epigenetic/transcriptional regulation

required to define spatial, temporal, and adaptive expression patterns of these genes may engage cell-lineage-specific TFs (Brohl et al. 2008; Huang et al. 2008; Wildner et al. 2013; Lu et al. 2014a), the insulators, and silencers such as CTCF and REST (Henriksson et al. 2014; Hou et al. 2008) along with mechanisms that control neuronal activity-dependent expression underlying neuronal plasticity. Understanding of the mechanisms that regulate epigenome and transcriptome in the neuropeptide-producing neurons is essential for understanding of physiology and pathology of neural circuits.

A specific feature of the neuropeptide genes is the presence in their body of short and repetitive neuropeptide sequences that also are highly conserved across species. In these properties the neuropeptide-encoding sequences are similar with DNA target elements for sequence-specific TFs. Interestingly, the dynorphin-encoding sequences are located in close proximity to or overlap with multiple transcription start sites, splice junctions, and also with CpG-SNP rs6045819 associated with alcoholism (Fig. 3.2b). They may be targeted by YY1 and NF-κB TFs and exert a promoter activity in reporter gene assay (Bakalkin et al. 1994, 1995, 1997; Nikoshkov et al. 2005). Furthermore, the dynorphin-encoding fragment encompasses the short CpG island, is hypersensitive to DNAase I (Fig. 3.2b), and adopts noncanonical, CpG methylation-dependent DNA secondary structures when present in single-stranded form (Taqi et al. 2012). The dynorphin sequences are also putative mutational hot spot; missense mutations located in this segment cause human neurodegenerative disorder SCA23 (Bakalkin et al. 2010; Jezierska et al. 2013; Saigoh et al. 2015). These mutations either create or destroy CpGs that represent differentially methylated sites; their methylation strongly differs between human brain and peripheral tissues, between brain areas, and between tissues and cultured cells suggesting their regulatory function (manuscript in preparation and (Bazov et al. 2011)). Altogether, these observations lend support to the hypothesis that the neuropeptide-encoding sequences are transcriptionally active; they may have a role in regulation of initiation and elongation of transcription and RNA splicing, all resulting in synthesis of canonical and/or novel transcripts that give rise to several variants of neuropeptide precursor proteins.

The most specific feature of neuron and neuronal circuit is its neuropeptide phenotype. The most specific and conservative feature of a neuropeptide gene is its neuropeptide-encoding sequences. These sequences may serve as DNA target elements for epigenetic and transcriptional mechanisms which define whether a specific gene is transcribed in a specific neuron. DNA signatures including (i) unique combination of canonical enhancer/promoter DNA elements and (ii) the neuropeptide-encoding sequences as the most specific gene identifiers may act in concert to mediate effects of cell-lineage-specific and activity-dependent TFs on transcription of neuropeptide genes. Whether epigenetic and transcriptional mechanisms target the neuropeptide-encoding sequences as signatures to define neuropeptide phenotype of multiple neuronal subtypes in millions of diverse neural circuits is a matter for future studies.

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Part II
Transgenerational Regulation of the Neuroendocrine System

Chapter 4
Epigenetic Risk Factors for Diseases: A Transgenerational Perspective

Johannes Bohacek and Isabelle M. Mansuy

Abstract Each individual is determined by a combination of genetic and non-genetic factors that together shape physiological and biological functions during development and adulthood. While genetic features are embedded in the DNA sequence inherited from parents, non-genetic features (which include epigenetic modifications) are acquired through experiences and environmental exposure across life. However, it is now acknowledged that non-genetic features can also be inherited from parents and propagate across generations. This chapter discusses the concept of non-genetic germline inheritance in mammals and examines possible routes of transmission of non-genetic information involving germline-dependent and germline-independent modes of transfer. It reviews current evidence that environmental factors can induce non-genetic alterations in the germline that can impact behavioral and physiological features in the offspring. This chapter also addresses the underlying molecular mechanisms, provides initial insight into the implication of epigenetic marks and non-coding RNAs in male germ cells, and questions the way non-genetic modifications can be induced and maintained in germ cells. It highlights promising areas of current research and reflects on evolutionary perspectives and future challenges.

Keywords Non-genetic germline inheritance • Epigenetics • Transgenerational Sperm • DNA methylation • Histone posttranslational modifications • ncRNAs

4.1 Introduction

In biology, inheritance is a process that ensures the transfer of phenotypic traits from parent to offspring. The most classic form of parental inheritance is genetic and involves genomic information in the form of DNA sequence in germ cells.

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However, non-genetic inheritance can also occur and critically contributes to the transmission of traits from parent to offspring (Bohacek and Mansuy 2013, 2015; Guerrero-Bosagna and Skinner 2012). In contrast to genetic inheritance, it does not involve DNA sequence per se but can nonetheless implicate the germline (Daxinger and Whitelaw 2012). Mounting evidence in the past few years has suggested that environmental factors can impact the non-genetic composition of germ cells and alter complex phenotypes including metabolism and behaviors involving mood, emotion, social behaviors, and cognitive processes in the offspring. This mode of inheritance is important for the field of medical genetics because it can provide an explanation for the missing heritability of many complex diseases such as psychiatric and metabolic disorders. These diseases are heritable, but their heritability cannot be explained by classic genetics and is clearly not associated with specific genes (Manolio et al. 2009; Eichler et al. 2010). Part of this missing heritability has been proposed to result from non-genetic mechanisms in the germline that allow environmental factors to impact behavior across generations (Bohacek and Mansuy 2013). Further, recent evidence has raised the possibility that epigenetic transgenerational effects may also play an important role in evolution by persistently modulating the genome and creating some genomic variability that can be maintained by natural selection (Skinner et al. 2014).

This chapter focuses on cases of non-genetic inheritance of alterations of behaviors or other functions induced by experiences or environmental factors. First, it reviews the non-genetic mechanisms present in mammalian germ cells, which represent potential vectors for the transmission of acquired information through the germline. Second, it describes in detail different behavioral and other phenotypes induced by various environmental factors and evidence for transgenerational transmission in humans and rodent models. Molecular components associated with these traits and behaviors are outlined when possible, and the potential existence of pathways of susceptibility is discussed. Finally, it discusses possible mechanisms underlying non-genetic alterations in the germline and the way they may be induced and maintained, and it elaborates on possible evolutionary implications of non-genetic germline inheritance.

4.1.1 What Is Non-genetic Germline Inheritance?

Inheritance in biology and psychology describes the transmission of characteristics and behaviors from parents to offspring. Germline inheritance is a biological concept that classically involves transmission of the genetic code by the germline. Traditionally, DNA has been considered the sole carrier of information that can be transmitted from parents to offspring. However, over the past decade, several non-genetic mechanisms possibly contributing to transgenerational germline inheritance have been proposed to operate in germ cells (Jenkins and Carrell 2012; Bohacek et al. 2012). These mechanisms comprise epigenetic modifications including DNA methylation (DNAme) and hydroxymethylation and posttranslational modifications

(PTMs) of histones and protamines, which together form the epigenome. They also include other non-genetic factors such as histone variants, nucleosome positioning, and noncoding RNAs (ncRNAs). For many of these mechanisms, there is evidence that they can be altered by environmental factors and by experiences. The idea that alterations of non-genetic factors induced by the environment can be inherited and impact the offspring is thus becoming mechanistically plausible (Bohacek and Mansuy 2013; Holland and Rakyan 2013; Gapp et al. 2014c). Therefore, non-genetic germline inheritance is a form of transmission of a feature or phenotype from parent to offspring that involves biological substrates other than the DNA sequence. Although independent from the DNA sequence, the substrates involved are tightly associated with the genome and can modulate its activity. Although it is termed non-genetic, this mode of inheritance implicates genes, but does not involve changes in their DNA sequence. The sum of non-genetic marks is generally more affected by the environment than the genome itself because non-genetic marks are inherently more vulnerable and susceptible to disruption, likely due to their labile nature. The rate of epimutations, a term originally introduced by Holliday to describe changes in DNA methylation that affect gene activity (Holliday 1987), is estimated to be orders of magnitude higher than for genetic mutations (Bennett-Baker et al. 2003). While cells have several active mechanisms to dynamically modify the epigenome, they have only a few to mutate the genome sequence, for instance, copy number variation or retrotransposition and immunoglobulin gene rearrangement. Further, although non-genetic marks can be modified, there are no known or specific active mechanisms of correction of alterations to these marks, while there are specific mechanisms of maintenance and repair of genetic integrity.

4.1.2 Complex Patterns of Non-genetic Germline Inheritance

Non-genetic germline inheritance relies on non-genetic modifications in the information carried by sperm cells or oocytes, in addition to the DNA sequence itself. Because they are embedded in germ cells, these modifications are often stable and can be transferred to the embryo at fertilization. In the offspring, these environmentally induced changes may occur again in germ cells and then be transmitted across subsequent generations, or these changes may no longer exist in the germline and not be perpetuated to subsequent generations. To differentiate these two possibilities, it is necessary to explore whether a specific alteration can be passed to the offspring and also to the grandoffspring. Notably, non-genetic inheritance can occur as well independently of germ cells. In this case, it is rather a transfer of acquired traits that requires continuous exposure to the causative factor at each generation for perpetuation. Such transmission is context dependent and involves behavioral, social, or environmental parameters. Several experimental strategies have been designed to distinguish germline-dependent and germline-independent non-genetic transmission in laboratory animals (see Appendix 1). Such strategies are however generally not applicable to human epidemiological studies.

In a given individual, non-genetic marks accumulate across life. They carry important information about the interactions of the individual with the environment and about the consequences of these interactions. Non-genetic changes transmitted to the offspring through the germline can have functional consequences at different stages of development and/or in adulthood, and resulting phenotypic manifestations may be apparent under normal conditions or may only be revealed under specific circumstances. To the offspring, they may confer an advantage when encountering a similar environment and give the offspring a head start. But if the environment is not the same, they may become maladaptive and no longer provide any benefit but even be disadvantageous. In extreme cases, they can increase disease susceptibility and favor psychiatric conditions, metabolic disorders, or cancer (Harper 2005; Jablonka and Raz 2009; Bohacek and Mansuy 2013). It may therefore be necessary to eliminate these acquired marks to give the new generation a clean start.

4.2 Potential Mechanisms of Non-genetic Germline Inheritance

Most work on germline-dependent transmission of non-genetic effects has focused on patrilines (offspring derived from males) and thus involves sperm cells. This avoids confounding maternal factors such as intrauterine environment and maternal care. However, the concept that acquired non-genetic marks can be inherited transgenerationally through the germline has been, until recently, fraught with major conceptual challenges: DNAme or histone PTMs (HPTMs) were not considered stable enough in sperm cells to allow transgenerational transmission. However, it is now accepted that epigenetic marks in germ cells are more stable and persistent than initially assumed. Further, it is now acknowledged that additional factors such as ncRNAs contained in sperm also play a role in non-genetic germline inheritance.

4.2.1 DNA Methylation

DNAme is prominent in sperm cells (80–90 % overall CpGs) and has a unique profile that is distinct from that in somatic cells or in oocytes (Oakes et al. 2007; Yamagata et al. 2007). Intergenic regions, CpG islands, and centric and pericentric chromosomal regions are differentially methylated such that genes involved in spermatogenesis are activated and genes involved in pluripotency and somatic functions are repressed. DNAme also exists in oocytes (about 40 %) and in the early embryo (Smallwood et al. 2011). DNAme in germ cells can be altered bidirectionally at different loci by environmental factors, and in some cases, the resulting epimutations are passed to the offspring. However, for DNAme marks in sperm to directly affect the offspring, they have to resist two rounds of reprogramming in the developing embryo, the first immediately after fertilization and the second between E10.5 and

E13.5. In the zygote after fertilization, DNA is massively demethylated (from 70 % to 30 %) by passive dilution by replication (Seisenberger et al. 2012; Lee et al. 2014) and by active 5mC to 5hmC conversion by TET dioxygenases in specific regions (Smith et al. 2012; Hackett et al. 2013). DNAme is then reestablished but erased again in developing germ cells at E10.5–E13.5. After this, only 7–14 % overall parental DNAme remain by the time of sex determination (Smith et al. 2012; Seisenberger et al. 2012). Starting in prospermatogonia around E15.5, DNAme is rapidly reacquired, and a germline-specific profile of DNAme is first fully established in pachytene spermatocytes after birth. These cycles are recapitulated in each pachytene spermatocyte generated in postnatal and adult life.

Despite overall reprogramming, DNAme can be maintained and escape reprogramming at some genetic loci. This is the case for imprinted genes (Sha 2008; Adalsteinsson and Ferguson-Smith 2014). Genomic imprinting is a mechanism that allows the silencing of one parental allele in a parent-of-origin-dependent manner. It results in a bias between the expression of parental alleles toward expression of the non-imprinted allele or even to strictly monoallelic expression. Therefore, by definition, imprinting is a form of transfer of epigenetic information from parents to offspring. Most imprinted genes (about 100 are currently known) contain imprint control regions (ICRs), which show differential DNAme between the maternal and the paternal allele. Sex-specific imprints are established during oogenesis and gametogenesis and retain their methylation profile during the wave of demethylation that occurs shortly after fertilization (Sha 2008; Adalsteinsson and Ferguson-Smith 2014). Further to imprinted genes, other sequences including intracisternal A particles (IAPs), retrovirus-like long-terminal repeat transposable elements also maintain their profile of DNAme in the mouse genome during embryonic development (Lane et al. 2003). Technological advances allowing genome-wide analyses of the dynamics of DNAme at high resolution during development have already led to the discovery of more regions across the genome where DNAme is not erased or only partially erased during embryogenesis (Borgel et al. 2010; Smith et al. 2012; Seisenberger et al. 2012). This combined evidence provides strong support that transmission of DNAme patterns in gametes can occur and may be an important mechanism for non-genetic transgenerational inheritance.

4.2.2 Histone Posttranslational Modifications

Similar to somatic cells, chromatin in sperm and oocytes also contains canonical histones and histone variants. However, in sperm, most histones are replaced by protamines, which are histone-like proteins that form toroid structures to tightly compact the genome. Only 1–5 % histones remain in mouse sperm (5–10 % in human) (Hammoud et al. 2009; Brykczynska et al. 2010). This likely results in the loss of epigenetic modifications that were carried by the removed histones although protamines were recently shown to also have PTMs in adult mouse sperm (Brunner et al. 2014). Recent studies also demonstrated that the retention of histones is tightly

regulated in sperm. However, the genomic pattern of nucleosome retention remains undetermined, and retention has been reported to be in hypomethylated regions, CpG islands, and gene promoters (including at developmental genes and microRNA clusters), but also in gene-poor regions (Hammoud et al. 2009; Brykczynska et al. 2010; Vavouri and Lehner 2011; Erkek et al. 2013; Carone et al. 2014; Samans et al. 2014). These discrepancies are likely due to technical limitations linked to the challenging methodologies used to study histone retention, such as immunoprecipitation and enzymatic digestion followed by deep sequencing (Casas and Vavouri 2014). Histones in sperm and oocyte show complex and distinct patterns of PTMs and likely reflect the different functional requirements of the genome in each cell type (Beaujean 2014). Sperm protamines carry PTMs such as lysine acetylation and methylation in mouse sperm (Brunner et al. 2014), which might play a role in genome regulation. Finally, different histone variants in sperm modulate nucleosomal dynamics by promoting interactions with chromatin remodeling complexes (Maze et al. 2014).

Even if histones are in low abundance in sperm chromatin, evidence suggests that they can be transferred to the oocyte after fertilization where they might play a critical role in embryonic development. In humans and mice, di- and trimethylated lysine 4 on histone 3 (H3K4me2, H3K4me3) and trimethylated lysine 27 on histone H3 (H3K27me3) are retained in sperm (Hammoud et al. 2009; Brykczynska et al. 2010; Erkek et al. 2013). H3K4me2 is associated with genes relevant for spermatogenesis and H3K27me3 with developmental regulatory genes. Further, there is also a correlation between H3K27me3 present at transcription start sites of genes in sperm and gene expression in early embryos, raising the possibility that this histone mark may contribute to paternal transmission of epigenetic information across generations, a possibility that needs to be tested (Brykczynska et al. 2010). Altogether, the highly regulated maintenance of histones in sperm and the presumed transmission of these histones and their PTMs to the zygote could play an important role in gene regulation in the developing embryo and allow transgenerational transmission of epigenetic information between parent and offspring.

4.2.3 Small ncRNAs in Sperm

Sperm cells contain various types of ncRNAs in germ granules including small and long ncRNAs and mRNAs. There are several populations of small ncRNAs: microRNAs, Piwi-interacting RNAs (piRNAs), small interfering RNAs, small nucleolar RNAs, mature sperm-enriched tRNA-derived small RNAs, etc. These ncRNAs are required for different processes including spermatogenesis, fertility, genome control (i.e., transposon silencing), and early development (Krawetz et al. 2011; Jodar et al. 2013). In human, a fertilizing sperm can bring an estimated 10–20 fg of RNA to the oocyte (Johnson et al. 2011) which may therefore also constitute a direct signal that carries information from one generation to the next.

MicroRNAs typically act to repress translation by targeting the 3′UTRs of their target mRNAs (Ghildiyal and Zamore 2009). miRNAs detected in mature sperm

mainly align to promoter regions; thus, they might bind paternal DNA and either recruit gene-silencing machinery (Kim et al. 2008), or prevent histone to protamine transition during sperm nuclear remodeling (Johnson et al. 2011). The role of sperm-delivered miRNAs in the oocyte remains unclear because endogenous miRNAs have been shown to only poorly repress target mRNAs in the oocyte (Ma et al. 2010). However, some studies have found that individual sperm-borne miRNAs, miR-135a and miR-34c, regulate key genes in the zygote and are crucial for normal embryo development (Pang et al. 2011; Liu et al. 2012). In addition, piRNAs are known to play a role in germ cell development, especially in the silencing of transposons by guiding DNAme (Aravin et al. 2008; Law and Jacobsen 2010). piRNAs can be detected in mature sperm in mice and humans (Krawetz et al. 2011; Gapp et al. 2014a); thus, if they are delivered from sperm to the zygote, they could play a role in establishing DNAme marks.

In mammals, several studies have highlighted the role of ncRNAs in germline inheritance. In a paramutation mouse model in which sperm RNAs are thought to induce fur depigmentation in wild-type offspring of mice heterozygous for a Kit mutation across generations, the injection of total sperm RNA from Kit mutants or of Kit-specific miR-221 and 222 into fertilized wild-type eggs recapitulates depigmentation (Rassoulzadegan et al. 2006). Other paramutation models of cardiac hypertrophy and altered growth could also be reproduced by injection of miR-1 and miR-124, respectively, into fertilized wild-type oocytes (Wagner et al. 2008; Grandjean et al. 2009). Recent work has shown for the first time that sperm RNAs can be altered by exposure to negative environmental conditions and transmit behavioral and metabolic alterations to the offspring across two generations (Gapp et al. 2014a). These data strongly suggest that RNA, RNA fragments, or microRNAs delivered from sperm to the egg upon fertilization can act as vectors of transmission of information across generations.

4.2.4 Technical Challenges Involving Non-genetic Germline Inheritance

While the presence of similar non-genetic modifications, i.e., changes in DNAme or ncRNAs, in the sperm of father and their offspring, and in different organs of the offspring, is a strong indication for transgenerational inheritance, more direct proof is required to demonstrate that these modifications are necessary for phenotypic expression and transmission. This is however conceptually and technically challenging. Thus, epimutations (changes in DNAme or HTPMs) cannot be easily reproduced at specific loci in a cell. However, fairly solid evidence for the role of epimutations has been provided by the Agouti and Axin(Fu) mouse models. In these models, the level of DNAme of an IAP retrotransposon in the Agouti and Axin(Fu) genes was shown to directly modulate gene expression and cause specific phenotypes transmitted to the progeny (Morgan et al. 1999; Rakyan et al. 2003). However, in most cases of transgenerational transmission, not only one but several loci or genes are affected, and identifying those responsible for the relevant phenotypes is

difficult. Further, alterations in sperm DNAme are usually small (a few percent) and hard to detect, partly due to the natural variability in DNAme in-between germ cells that can occlude the detection of epimutations (Court et al. 2014). Similarly, a large number of known HPTMs exist, their effects are locus specific, and they interact with each other in a complex manner (Lee et al. 2010). Causal evidence is also limited by the difficulty in linking DNAme/HPTMs to gene expression in sperm cells since these cells are transcriptionally silent. Most epimutations are nonfunctional in sperm and might be maintained and/or relayed by other marks to modulate gene activity later in development or adulthood. Assigning epimutations to gene activity is also problematic because some genes are regulated by epigenetic marks in distal regions (e.g., enhancer regions) with no obvious physical link. In this respect, confirming that epimutations are functional requires identifying the target genes, cells, and time window in which they control gene activity. Although epimutations transmitted by sperm should affect every cell in the progeny, they may be inefficiently maintained or corrected during development and be present only in some cells in the offspring. An ultimate proof for their causal contribution to transmission would require mimicking them at individual loci or even CpGs in germ cells, which is technically extremely challenging but may be possible in the future with the development of novel techniques using CRISPR-Cas9 or zinc-finger proteins (Heller et al. 2014; Hilton et al. 2015). In contrast to epimutations, mimicking alterations in sperm RNAs is doable. Thus, sperm RNAs can be extracted and injected into fertilized oocytes in an attempt to reproduce their effects after normal fertilization and to causally test their involvement in germline transmission (see Sect. 4.2.3).

4.3 Current Evidence for Germline Inheritance of Traits and Behaviors Induced by Experiences and Environmental Exposure

Environmental factors and events encountered acutely or chronically during life can modify biological functions and behavior in the exposed individual transiently or permanently, depending on the nature, strength, and duration of exposure. In addition to affecting individuals that are directly exposed, some factors or events can also impact the progeny and have consequences on individuals across several generations. This section summarizes the evidence for such non-genetic germline inheritance in mammals and describes various biological functions including brain functions, behavior, and metabolic responses that have been implicated.

4.3.1 The Case for Transgenerational Epigenetic Inheritance in Humans

Due to conceptual, technical, and ethical limitations in human studies, there is currently no causal evidence for non-genetic germline inheritance in humans. The best evidence for transgenerational inheritance induced by environmental factors in

humans relates to the effects of diet and food availability in early life. Children, in particular boys, exposed *in utero* to the 5-month Dutch famine in 1944/1945 seem to be at higher risk to develop a host of clinical disorders ranging from obesity to glucose intolerance and coronary heart disease when adult (Susser and Stein 1994; Kyle and Pichard 2006). In some subjects, the symptoms could be associated with altered DNAme in the imprinted IGF2 gene in blood 60 years later (Heijmans et al. 2008). The children of these subjects also appear to develop pathological symptoms and have higher adiposity at birth and poorer health than the offspring of unexposed fathers (Painter et al. 2008; Veenendaal et al. 2013). The risks seems to be the highest when gestational famine was followed by consumption of calorie-rich diet later in life, suggesting an effect of mismatch conditions (Schulz 2010). However, different results on the offspring's birth weight have been reported by other groups, suggesting a possible high variability in cohorts or an inconsistency in the measured effects (Lumey and Stein 1997; Stein and Lumey 2000). In two unrelated cohorts, food supply during the slow-growth period was shown to affect health in the grandoffspring. While abundant food supply in paternal grandmother or grandfather during prepuberty increases mortality risk due to cardiovascular disease and diabetes in granddaughters and grandsons, respectively (Pembrey et al. 2006; Kaati et al. 2007), poor food supply in prepuberty reduces mortality risk in the grandoffspring (Pembrey 2010; Van Den Berg and Pinger 2014). Diet variation depending on season of conception has also been reported to affect health and DNAme in first-generation offspring in Gambia (Waterland et al. 2010). Other studies have reported the effects of nutritional or environmental factors such as nutritious food supplementation, paternal smoking in childhood, or maternal or paternal stress exposure, from parent to offspring in humans (Engel et al. 2005; Chen et al. 2006; Behrman et al. 2009; Vaage et al. 2011; Schick et al. 2013). Importantly, all studies in humans are inevitably confounded by genetic heterogeneity, family dynamics, living and seasonal conditions, and poor experimental control, all of which need to be carefully considered when interpreting results (Lumey et al. 2011). These experimental factors can be much better controlled in mammalian model systems used in laboratory settings, particularly in rodents (rats and mice), which will be reviewed below.

4.3.2 Evidence for Inheritance of Acquired Behaviors in Laboratory Animals

Over the past decade, many studies in mammalian model systems have documented cases of non-genetic germline inheritance involving metabolism, brain functions, and complex behaviors. Rodent models have the unique advantage of providing several experimental controls including the use of isogenic mice, short reproductive cycles, and the possibility to apply assisted reproductive techniques like *in vitro* fertilization (IVF) to test germline dependence of transgenerational effects (see Appendix 1). In addition, tissue samples can be collected at selected time points during the experiments, including embryos and germ cells, to detect non-genetic alterations. The following section reviews the currently available evidence in

support of non-genetic germline inheritance of acquired behaviors by focusing on overlapping behavioral/metabolic phenotypes induced by various environmental factors. This is complemented by a tabular overview that sorts studies based on environmental factors/experimental manipulations, highlighting the extent of the currently available data and the complexity of observed sex differences, which remain poorly understood (Table 4.1). The extensive literature on transgenerational effects of endocrine disruptors and environmental toxins is not included in the table, and readers are referred to recent reviews on these models (Nilsson and Skinner 2014; Skinner 2014) and on metastable epialleles (Daxinger and Whitelaw 2012). Also, studies using matrilines and that assess only the offspring (F1) but not grandoffspring (F2) while not controlling for the potential impact of maternal behaviors (see Appendix 1) are not considered.

4.3.2.1 Metabolic Functions

Over the past few years, many studies have reported pronounced diet-induced effects on metabolic functions across generations in animals. Slightly different paradigms in which rats or mice are starved or overfed before or during breeding and gestation result in remarkable transgenerational phenotypes.

Undernutrition Undernutrition induced by 50 % caloric restriction during the last week of gestation in mice alters metabolic functions in the offspring of exposed mice. It reduces body weight, alters glucose and insulin metabolism, and changes gene expression in the liver and pancreas (Jimenez-Chillaron et al. 2009; Carone et al. 2010; Martínez et al. 2014; Radford et al. 2014). Although a causal relationship with non-genetic marks is difficult to establish (see Sect. 4.3.1), differences in DNAme are found across the genome in sperm of adult mice exposed to undernutrition *in utero* (Radford et al. 2014), and in some cases these changes coincide with changes in gene expression (Martínez et al. 2014; Radford et al. 2014).

Overfeeding Overfeeding or high-fat diet in male rats and mice can lead to marked transgenerational alterations in glucose/insulin metabolism that are coupled with widespread changes in gene expression in the pancreas (Dunn and Bale 2009, 2011; Pentinat et al. 2010; Ng et al. 2010; Wei et al. 2014). In some cases, these alterations can be very persistent and be observed in the grandoffspring of exposed males (Pentinat et al. 2010; Wei et al. 2014). Changes may be due to alterations in DNAme in the sperm of fathers, particularly since these changes can still be observed in early post-implantation embryos, suggesting that the marks in germline can resist DNAme reprogramming in the zygote (Wei et al. 2014).

Stress and Toxins Interestingly, alterations in glucose/insulin metabolism have also been reported in male mice exposed to postnatal traumatic stress and in their offspring (Gapp et al. 2014a). In this case, transmission could be reproduced by injecting sperm RNAs collected from stress-exposed males into fertilized control oocytes, but also in the progeny of the resulting animals, suggesting a causal

Table 4.1 Transgenerational effects of environmental factors in rodents

Model	Species	Breeding design	Tests/assays	Effect in sperm[a]	Effect in F1 (yes/no)	Effect in F2 (yes/no)	Sex differences	References
Stress/trauma models								
Unpredictable maternal separation combined with unpredictable maternal stress 3 h/day, from postnatal days (PND) 1–14. Exposed pups = F0	Mouse	P	FST/SPT/TST/FEP/OFT		Y/N/Y/Y/Y	Y/N/Y/Y/Y	Complex interaction	Franklin et al. (2010)
			Hippocampus: GE/DNAme	Y (DNAme)	Y/Y	Y (sperm)	Males tested	
			Social interaction/social memory/social defeat		Y/Y/Y	Y/Y/n.a.	Complex interaction	Franklin et al. (2011)
			Serotonin receptor binding (brain)	n.a.	Y	n.a.	Males tested	
		P(+sperm RNA inj.[b])	EPM/light-dark box/FST/blood glucose/blood insulin		Y/Y/Y/Y/Y	n.a.	Males tested	Gapp et al. (2014a)
			Hippocampus miRNAs	Y	Y	N		
		P(+cross-fostering)	FST/FC/NOR		Y/Y/Y	n.a./N/n.a.	Females tested	Bohacek et al. (2015)
			Hippocampus: GE/DNAme	Y (DNAme)	Y/Y	N/N		
			LTP/LTD		Y/Y	N/N	No	
		P	EPM/operant conditioning tasks		Y/Y	n.a.	No	Gapp et al. (2014b)
			Hippocampus: GE/HPTMs/DNAme	Y (DNAme)	Y/Y/N	n.a.	Males tested	
		M(+cross-fostering)	FEP/EPM		Y/Y	n.a.	No	Weiss et al. (2011)

(continued)

Table 4.1 (continued)

Model	Species	Breeding design	Tests/assays	Effect in sperm[a]	Effect in F1 (yes/no)	Effect in F2 (yes/no)	Sex differences	References
Chronic social instability (7 weeks) starting PND 27. Exposed mice = F0	Mouse	M, P, MxP	EPM/OFT/social interaction/ sociability/social memory		Y/Y/Y/N/Y	Y/Y/Y/ Y/Y	F1/F2/F3 effects only in females	Saavedra-Rodriguez and Feig (2013)
			Hippocampus GE		Y	Y		
Chronic variable stress (1st gestational week). Exposed fetuses = F0	Mouse	P	Anogenital distance/testis weights/TST/BM/plasma CORT		Y/Y/Y/N/N	n.a.	F1 effects only in males	Morgan and Bale (2011)
			Brain postnatal day 1: GE, miRNAs	n.a.	Y/Y	n.a.		
Social defeat stress (10d) in adulthood. Exposed males = F0	Mouse	P(+IVF)	Submaximal chronic defeat/ EPM/NMA/SPT/FST		Y/Y/Y/Y/Y	n.a.	NMA + SPT + VEGF effects only in males; only EPM effect stays after IVF	Dietz et al. (2011)
			Plasma: corticosterone, VEGF	n.a.	Y/Y	n.a.		
Chronic variable stress 42d, puberty/ adulthood. Exposed males = F0	Mouse	P	Prepulse inhibition/TST/BM/ LDB/plasma CORT		N/N/N/N/ N/Y	n.a.	No	Rodgers et al. (2013)
Drugs of addiction								
Chronic ethanol vapor exposure (5 weeks) in adult males. Exposed males = F0	Mouse	P	Weight, EtOH preference, EPM[c], OFT, rotarod, EtOH clearance test		Y/Y/Y/N/ N/N	n.a.	Effects only in males	Finegersh and Homanics (2014)
			VTA (brain): GE, DNAme	Y (DNAme)			No	

Fetal ethanol exposure (gestation days 7–21). Exposed embryos = F0	Rat	P,M	ARC (brain): GE/DNAme	Y (DNAme) in F1 and F2 sperm	Y/Y	Y/Y	F1/F2 GE effects are male specific	Govorko et al. (2012)
Cocaine self-admin. (60d) in adult males. Exposed males = F0	Rat	P	Cocaine intake (self-administration)		Y	n.a.	Effects only in males	Vassoler et al. (2013)
			mPFC (brain): GE, HTPM	Y (HPTMs)	Y	n.a.		
Adult chronic morphine exposure (10d). Exposed animals = F0	Rat	P,M,MxP	EPM/OFT/water maze/ dendritic morphology hippocampus/ GE hippocampus		Y/Y/N/Y/Y	n.a.	No	Li et al. (2014)
Adolescent chronic morphine exposure (10d). Exposed female pups = F0	Rat	M	Dopamine agonist-induced locomotor activity/plasma corticosterone/GE nucleus accumbens (brain)		Y/Y/Y	Y/Y/Y	Only males tested	Byrnes et al. (2013)
Chronic nicotine (embryonic day 6 to PND21). Exposed fetuses/pups = F0	Rat	MxP	Respiratory system resistance and compliance/tracheal restriction/GE in lung fibroblasts		Y/Y/Y	Y/Y/Y	Stronger effects in males	Rehan et al. (2013)
Others								
Enriched environment from PND15 to PND29. Exposed pups = F0	Mouse	P, M(cross-fostering)	LTP/FC		Y/Y	N/N	No, but effects passed only through matriline	Arai et al. (2009)

(continued)

Table 4.1 (continued)

Model	Species	Breeding design	Tests/assays	Effect in sperm[a]	Effect in F1 (yes/no)	Effect in F2 (yes/no)	Sex differences	References
Lipopolysaccharide challenge (PND1 + 3). Exposed pups = F0	Rat	P, M, M(+cross-fostering)	EPM/hole-board test/ acoustic startle/corticosterone		Y/Y/N/N	n.a.	Complex differences btw. matri-/patriline	Walker et al. (2012)
Olfactory fear conditioning in adult males. Exposed males = F0	Mouse	P(+IVF), M(+cross-fostering)	Odor potentiated startle/ odor-specific glomeruli		Y/Y	Y/Y	Males tested	Dias and Ressler (2014)
			DNAme (sperm)	Y	n.a.	n.a.		
Undernutrition								
50 % caloric restriction during last week of gestation. Exposed fetuses = F0	Mouse	M, P, MxP	Birth weight/GTT/ITT		Y/Y/Y	n.a.	Males tested	Jimenez-Chillaron et al. (2009); Martínez et al. (2014); Radford et al. (2014)
			GE: pancreas/adipocytes/liver/ brain		Y/Y/Y/Y	n.a.		
			DNAme	Y	Y	n.a.		
Low-protein diet from weaning until sexual maturity. Exposed animals = F0	Mouse	P	Liver: GE/ miRNAs/DNAme	N	Y/Y/Y	n.a.	Males tested	Carone et al. (2010)
			HPTMs	Y	n.a.			
Overnutrition								
Dams fed high-fat diet from 6 weeks before breeding until weaning. Exposed fetuses = F0	Mouse	M, P, MxP	Body length		Y	Y	F2 effect only in females	Dunn and Bale (2009, 2011)
			GTT/ITT		N/Y	Y/N		
			Plasma leptin		Y	n.a.	No	

Overnutrition by reducing litter size to 4 pups. Exposed pups = F0	Mouse	P	Blood glucose/insulin/ triglycerides		Y/Y/Y	Y/N/N	Males tested	Pentinat et al. (2010)
			GTT		Y	Y		
High-fat diet in males from weaning until mating. Exposed rats = F0	Rat	P	GTT		Y	n.a.	F1 effect only in females	Ng et al. (2010)
			Pancreatic islets: GE/DNAme	n.a.	Y/Y	n.a.	Females tested	
High-fat diet in males from weaning + injection of hepatotoxin (STZ). Exposed mice = F0	Mouse	P	GTT/ITT		Y/Y	Y/Y	No	Wei et al. (2014)
			Pancreatic islets: GE/DNAme	Y (DNAme)	Y/Y	Y/Y	No	

F0 parent generation, *F1* offspring, *F2* grandoffspring, *n.a.* data not available. **Breeding design:** *M* matriline, P patriline, MxP both lines crossed. **Tests/assays:** *GE* gene expression, *DNAme* DNA methylation, *HPTMs* histone posttranslational modifications, *FST* forced swim test, *SPT* sucrose preference test, *TST* tail suspension test, *FEP* free exploratory paradigm, *OFT* open-field test, *EPM* elevated plus maze, *LDB* light-dark box, *FC* fear conditioning, *NOR* novel object recognition, *LTP* long-term potentiation, *LTD* long-term depression, *BM* Barnes maze, *CORT* corticosterone, *NMA* novelty motor activity, *VTA* ventral tegmental area, *ARC* arcuate nucleus (hypothalamus), *mPFC* medial prefrontal cortex, *GTT* glucose tolerance test, *ITT* insulin tolerance test

[a]Effect in sperm (yes/no), if applicable

[b]Sperm RNA was injected into fertilized oocytes

[c]EPM after acute EtOH injection

implication for sperm RNAs in the observed metabolic phenotypes and their transmission. Additionally, *in utero* exposure to the insecticide dichlorodiphenyltrichloroethane strongly increases the incidence of obesity in the grandoffspring of exposed male and female rats (Manikkam et al. 2013). Altogether, these findings suggest that there are various factors that can regulate body weight, metabolism, and glucose/insulin signaling in a transgenerational manner. This is in line with the human data (see Sect. 4.3.1) that suggest a relationship between ancestral food intake and health of the subsequent generations.

4.3.2.2 Social Behaviors

Stress Exposure to traumatic events during early postnatal life can alter social behaviors across generations in mice. When male mice are exposed to unpredictable maternal separation combined with unpredictable maternal stress (MSUS) daily from birth to postnatal day 14 (PND14), their male offspring and grandoffspring show reduced social interaction/sociability. In female offspring and grandoffspring, social recognition memory is impaired, while olfactory recognition memory is intact, suggesting impairments specific to social interactions (Franklin et al. 2011). Similarly, chronic social instability resulting from frequently changing cage mates during adolscence (PND27–76) reduces social interactions and preference for a novel conspecific symptoms that are transmitted across three subsequent generations by both females and males (Saavedra-Rodriguez and Feig 2013). In both models, males can be silent carriers as asymptomatic fathers sire offspring with altered social behaviors (Franklin et al. 2011; Saavedra-Rodriguez and Feig 2013). Across generations, males seem to pass the behavioral alterations to the offspring more faithfully than females (Saavedra-Rodriguez and Feig 2013). Notably, different neuronal alterations have been identified in the different models. Serotonergic signaling is disturbed in the offspring of stressed fathers; 5HT1A receptor binding is decreased in several brain regions including the CA1 subregion of the hippocampus and the dorsal raphe (DR) of the affected offspring (Franklin et al. 2011). Concomitant with reduced 5HT1A autoreceptor binding in the DR, 5HT release is increased in the frontal cortex, one of DR target projection areas. Expression of the calcineurin inhibitor gene Rcan1/2 is also decreased in hippocampus area CA1 across three subsequent generations (Saavedra-Rodriguez and Feig 2013). Serotonergic signaling and Rcan1 expression are functionally linked (Hoeffer et al. 2013), suggesting that the similarities between social alterations in both studies may share overlapping molecular signaling mechanisms. Other studies using various chronic stress models in rats and monkeys have noted effects of social- and stress-related responses in the offspring (Kinnally et al. 2013; Babb et al. 2014). However, as the matriline was studied without controlling for maternal care, the results may be confounded by maternal effects and are therefore not informative with regard to germline non-genetic inheritance (see Appendix 1).

Chemicals *In utero* exposure to the endocrine disruptor bisphenol A (BPA), a synthetic compound used to make certain plastics, reduces social interactions in exposed mice,

but leads to a transgenerational increase in social behaviors in several subsequent generations bred through the male line (Wolstenholme et al. 2012, 2013). BPA also leads to a subtle transgenerational impairment in recognizing social novelty (Wolstenholme et al. 2013). In the embryonic brain (E18.5), transgenerational reduction of arginine vasopressin gene expression was detected, suggesting that hormonal signaling may contribute to inheritance of altered social behaviors (Wolstenholme et al. 2012).

4.3.2.3 Stress Responsiveness and Anxiety-Related Behaviors

Stress During Early Postnatal Life Chronic unpredictable traumatic stress (involving maternal separation) during the first 2 weeks of postnatal life alters the response to aversive situations in adulthood. The mice subjected to postnatal traumatic stress enter unfamiliar compartments of a cage more readily and explore more readily the aversive center of an open field (bright and unsafe) or the open and unprotected arms of an elevated plus maze than controls. These alterations are passed to the offspring and grandoffspring through both males and females (Franklin et al. 2010; Weiss et al. 2011; Gapp et al. 2014a, b). In the female line, behavioral changes in the offspring persist even after cross-fostering (Weiss et al. 2011). In the male line, marked alterations in small ncRNA in sperm were detected in exposed fathers by next-generation sequencing (Gapp et al. 2014a). When RNAs were extracted from the sperm of these fathers and microinjected into wild-type fertilized mouse oocytes, the same behavioral alterations were observed in the animals arising from RNA-injected oocytes but also in the progeny of these animals. This provides causal evidence that sperm RNAs are key vectors of transmission of the effects of early stress to the offspring.

Stress During Adolescence/Adulthood Chronic variable stress experienced for 42 consecutive days starting either during adolescence or in adulthood also induces changes in sperm microRNA composition, suggesting that sperm RNAs can be altered throughout the lifespan (Rodgers et al. 2013). However, stress in adulthood does not have effects in the offspring except for slightly lower levels of corticosterone at baseline and after stress and altered expression of some genes in stress-related brain regions. This is in contrast with mice subjected to chronic social stress starting in adolescence, which dramatically increases anxiety across several subsequent generations (Saavedra-Rodriguez and Feig 2013). Only females express the increased anxiety, but with remarkable consistency across at least four generations. While stress-exposed parents can both transmit the anxiety phenotype to their first-generation offspring, only males pass the effects to subsequent generations. This is despite the fact that they, themselves, have no change in anxiety. This suggests that any changes induced by stress in the germline – in this model – can be erased in oocytes in the second generation, while this resetting does not seem to occur in sperm cells across several generations.

Adult male mice exposed to chronic social defeat for 10 days (1 month before mating) sire offspring with increased anxiety (Dietz et al. 2011). However, this

increase is not observed in animals generated by *in vitro* fertilization (IVF), implying that the effects are germline independent. However, it is also possible that IVF, known to alter epigenetic programming, interferes with non-genetic marks necessary for transmission (see Appendix 1) (Denomme and Mann 2012). It is notable that the various chronic stress paradigms used in mice or rats have different behavioral outcomes in the offspring, suggesting that the severity and timing of stress are likely critical factors determining whether alterations are induced and transmitted. The molecular mechanisms underlying such complex and specific effects remain unknown.

Drugs and Chemicals Fetal alcohol exposure in rats leads to heightened stress-axis sensitivity in the exposed animals and in their male offspring and grandoffspring (Govorko et al. 2012). In these animals, plasma ACTH and corticosterone levels are higher than control levels both at baseline and following an immune challenge. Across generations, proopiomelanocortin (POMC) expression in the arcuate nucleus of the hypothalamus was reduced (but not in the nearby paraventricular nucleus), together with increased DNAme and reduced DNA hydroxymethylation at the POMC promoter. Changes in DNAme were also detected in the sperm in three subsequent generations (Govorko et al. 2012). Administration of the bacterial mimetic lipopolysaccharide to rats on postnatal days 3 and 5 leads to anxiety-like behavior in males (but not females) later in life (Walker et al. 2012). Their offspring similarly show increased anxiety and more risk-assessment behaviors transmitted both through mothers and fathers. As the matriline effects can be reversed by cross-fostering, it remains to be shown that transmission through the patriline is indeed germline dependent. Finally, *in utero* exposure to high doses of the agricultural fungicide vinclozolin, a potent endocrine disruptor, leads to many dramatic and persistent transgenerational molecular changes (Guerrero-Bosagna and Skinner 2012; Skinner 2014). Behaviorally, the grandoffspring of rats exposed *in utero* to vinclozolin have altered anxiety (Skinner et al. 2008). However, this effect could not be replicated (Gillette et al. 2014), and the authors suggest that multiple "hits" may accumulate over generations and affect anxiety responses. Indeed, when the grandoffspring of vinclozolin-treated males are exposed to chronic stress in adolescence, complex interactions emerged, such that depending on sex and grand-paternal vinclozolin history, anxiety measures showed subtle alterations compared to controls. These effects need to be confirmed and maternal effects need to be better controlled. Finally, 10 days of morphine exposure in male and female rats leads to strong anxiety behavior in the offspring that is transmitted through both the matriline and patriline (Li et al. 2014). This effect is dependent on reduced IGF-2 signaling in the hippocampus and can be rescued by IGF-2 overexpression and by exposing the offspring to enriched environmental conditions after weaning. Similarly, early life stress induces anxiety in the offspring that can be reversed by parental exposure to environmental enrichment (Leshem and Schulkin 2012) although these effects were only tested in the female line, and may thus not be germline-dependent (see Appendix 1).

4.3.2.4 Depressive Symptoms

Depression in humans is a complex syndrome resulting in multiple behavioral dysfunctions. In animal models, specific aspects of depressive behaviors can be induced by, for instance, social isolation, and some behavioral manifestations like behavioral despair and anhedonia can be measured (Porsolt et al. 1977). Several studies in mice have demonstrated a link between chronic stress experienced at different developmental stages and increased behavioral despair in the offspring (Mueller and Bale 2008; Franklin et al. 2010; Dietz et al. 2011; Gapp et al. 2014a; Bohacek et al. 2015).

Prenatal Stress Exposure of females to chronic stress during the first week of gestation increases immobility on the tail suspension test in the exposed males and their male offspring (Mueller and Bale 2008; Morgan and Bale 2011). In perinatal life, the male offspring have a general shift in gene expression and miRNAs content toward a female-like expression pattern in the brain. Several miRNAs are increased, and one target, beta-glycan (a TGF-beta-associated gene), has increased expression in the early postnatal brain (Morgan and Bale 2011). It remains to be determined if this effect involves the germline and can be transmitted to the grandoffspring.

Postnatal Stress Chronic traumatic stress increases behavioral despair (immobility) on both forced swim and tail suspension tests in adulthood. This effect is transmitted to the offspring and grandoffspring by males in a sex-dependent manner, with female offspring and male grandoffspring showing increased immobility (Franklin et al. 2010). In sperm cells of stressed fathers and their offspring, DNA is hypo- or hypermethylated at the promoter regions of several candidate genes involved in stress, emotionality, or epigenetic regulation, including corticotrophin-releasing factor receptor 2 (*Crfr2*, also *Crhr2*), cannabinoid receptor 1 (*Cb1*), and methyl-CpG binding protein 2 (*Mecp2*). Similar alterations in DNAme and parallel changes in gene expression also affect the brain of the F2 progeny (Franklin et al. 2010). In addition to changes in DNAme, the sperm RNA profile is altered in stressed males, and injection of these sperm RNAs into fertilized control oocytes can recapitulate the depressive-like behaviors in the resulting animals (Gapp et al. 2014a). While this demonstrates germline-dependent non-genetic transmission, it also suggests that changes in DNAme in sperm may not be directly required for transmission of the depressive phenotype. However, DNAme may be involved in the downstream consequences of RNA alterations, for instance, some of the altered ncRNAs may be regulators of DNMTs or other members of the epigenetic machinery. Interestingly, while the miRNA composition is altered in sperm of exposed males, it is not altered in the sperm of the offspring (Gapp et al. 2014a). Nonetheless, the offspring still transmits depressive-like behaviors to the grandoffspring (Franklin et al. 2010), raising the possibly that DNAme, which unlike miRNAs remains altered in sperm of the F2 offspring of exposed males, may be essential for transmitting the effects to subsequent generations. These hypotheses need to be tested in the future.

Stress in Adulthood Chronic social defeat in adult male mice 1 month before mating increases immobility in the forced swim test in the male and female offspring. The effect is maintained in animals generated by IVF using sperm of the stressed males (Dietz et al. 2011). This again strongly supports transmission of acquired depressive-like behaviors through the germline. In this model, it would be useful to test whether sperm RNAs are involved in transmission such as demonstrated after early postnatal stress (Gapp et al. 2014a), or whether the mechanisms differ between stressors experienced during different developmental time windows, in particular in adulthood when germ cells should be less vulnerable than during development. Although different stressors can have similar effects across generations, there are notable differences in the sex specificity of the observed effects, yet the reason for this remains unknown. Interestingly, chronic stress exposure during adolescence or adulthood does not have any transgenerational effects on behavioral despair (Saavedra-Rodriguez and Feig 2013; Rodgers et al. 2013), suggesting that the severity and timing of the stressor likely determines its transgenerational impact. More studies of the underlying mechanisms are required to clarify this possibility.

4.3.2.5 Drug Preference and Related Behaviors

Several studies have reported transgenerational effects of drugs of abuse in animal models.

Cocaine When adult male rats are allowed to self-administer cocaine for 60 days, their male (but not female) offspring acquire cocaine self-administration more slowly and have reduced total cocaine intake (Vassoler et al. 2013). The male offspring of cocaine-treated sires express more brain-derived neurotrophic factor (BDNF) in the medial prefrontal cortex (mPFC), associated with increased histone 3 acetylation (AcH3) at BDNF promoter IV. Similarly, AcH3 is enriched at the BDNF promoter in the sperm of their father, suggesting that cocaine may alter sperm chromatin, an effect passed to the offspring. However, it remains to be established whether this phenotype is also transmitted to the grandoffspring, and whether a change in HPTMs in sperm is sufficient to mediate transmission, considering that sperm cells maintain only 1–5 % histones and that sperm H3 acetylation is undetectable in mice (Brunner et al. 2014).

Alcohol Chronic paternal exposure to ethanol vapors (8 h/day for 5 weeks) immediately before mating reduces ethanol preference and total ethanol consumption in the male (but not the female) offspring in mice (Finegersh and Homanics 2014). The offspring of ethanol-treated fathers also show a strong anxiolytic response on the elevated plus maze in response to ethanol injections, an effect not observed in controls. Their motor performance after ethanol injections, however, is better than in control offspring. Further, ethanol-exposed fathers have decreased body weight, but their male offspring have increased body weight. DNAme is decreased at the BDNF promoter in the sperm of ethanol-exposed fathers and the brain of the offspring, and BDNF is higher in the ventral tegmental area (but not PFC) of the offspring.

In support of lasting epigenetic changes induced by alcohol in the germline, another study reported locus-specific changes of DNAme in sperm of three subsequent generations after an initial *in utero* ethanol exposure (Govorko et al. 2012).

Opioids Ten days of repeated morphine injections during adolescence in female rats increase the sensitivity of the male offspring to the analgesic effects of morphine (Byrnes et al. 2011). Further, locomotor activity induced by repeated administration of the D2/D3 dopamine receptor agonist quinpirole is reduced in the male offspring and grandoffspring (Byrnes et al. 2013). In response to quinpirole, the male offspring have higher corticosterone release and increased expression of the D2 receptor mRNA in nucleus accumbens. Since maternal confounds cannot be excluded, the interpretation of these results is limited. However, the observations that chronic parental drug exposure can decrease the sensitivity of the offspring to the same (or possibly related) drug fit with the cocaine and ethanol data presented above and suggest that parental exposure induces a form of drug resistance. This is in contrast to epidemiological studies in humans, which show that drug addiction often runs in families and is passed from parent to children (Merikangas et al. 2014). In animals, it is possible that there is a natural response to counteract alterations of neuronal mechanisms induced by drug exposure, in a way to prepare the offspring to better cope with subsequent exposure. But in humans, the psychosocial/socioeconomic component associated with parental drug abuse and its impact on family dynamics might obscure such adaptive effects.

Nicotine *In utero* exposure to nicotine in rat, based on a model of maternal smoking, induces asthma-like impairments in lung functions in the grandoffspring, with stronger effects in males than females (Rehan et al. 2013). In this model, since the grandoffspring was obtained by cross-breeding male and female offspring from the exposed mother, it is unclear whether transmission is germline dependent or not, and whether both the male and female germline are affected. But the results support human epidemiological data suggesting that nicotine exposure during pregnancy can increase asthma risk across generations (Li et al. 2005).

4.3.2.6 Cognitive Functions

Diet Several studies in the mid-1970s reported that rats fed low-protein diet across many generations have complex behavioral alterations such as deficits in home-orienting behavior and visual discrimination and that these effects are more pronounced than when rats are malnourished for only one generation (Stewart et al. 1975; Galler 1980, 1981). When returning the malnourished animals to a normal diet for two generations, some of the deficits disappear, but others persist (Stewart et al. 1980; Galler and Seelig 1981; Galler 1981). The reversibility of some of the effects suggests no genetic change; however, the effects that persisted after restoration of normal diet may have been contributed for by genetic alterations.

Postnatal Stress Severe stress during the first 2 weeks of postnatal life impairs social recognition memory in the offspring and grandoffspring of exposed males (Franklin et al. 2011). Further, hippocampus-dependent spatial memory is also impaired in stressed fathers and their offspring, an effect that is accompanied by a dramatic shift in synaptic plasticity in the hippocampus (Bohacek et al. 2015). Long-term potentiation (LTP), a form of synaptic plasticity reflecting synaptic strengthening, is abolished in the stressed fathers and their offspring, while long-term depression (LTD), a form of synaptic weakening, is enhanced. Widespread changes in gene expression occur in the hippocampus of the offspring, where molecular pathways related to synaptic transmission and calcium signaling are suppressed. For example, expression of the brain-specific gamma isoform of protein kinase C (*Prkcc*) is reduced in the hippocampus of the offspring, and DNAme at the *Prkcc* promoter is altered both in the brain of the offspring and the germline of the stressed fathers. The effects are transmitted through males and persist after cross-fostering, but are not passed to the grandoffspring (Bohacek et al. 2015).

But early life stress can also have positive effects on cognitive functions across generations. On a goal-directed task assessing an animal's ability to respond at a fixed delay to receive a reward, the offspring of stressed fathers perform better than controls (Gapp et al. 2014b). The offspring also outperform controls in an operant conditioning test when task contingencies suddenly change to achieve a fixed goal (get a reward), suggesting improved behavioral flexibility. Similarly, during extinction, the offspring of stressed fathers also stop responding more readily, suggesting less perseveration. The level of mRNA and protein expression of the mineralocorticoid receptor (MR), known to be involved in mediating behavioral flexibility, are lower in the hippocampus of exposed fathers and their offspring. Consistently, chronic administration of the MR antagonist spironolactone to the offspring can reverse some of the behavioral changes, suggesting a causal involvement of MR signaling. In the hippocampus of the offspring, while DNAme at the MR promoter is not affected, several histone modifications known to be associated with transcriptional activation are reduced and correlate with lower MR expression. Interestingly, changes in DNAme at the MR promoter are observed in sperm of the exposed fathers, suggesting that DNAme may play a role in transmission of the acquired phenotype (Gapp et al. 2014b).

Environmental Enrichment Stimulating environmental conditions, particularly early in life, can have positive effects on cognitive functions, and some of these effects can act across generations. Exposing mice to enriched environmental conditions during adolescence improves memory performance and increases synaptic plasticity, in particular LTP in the hippocampus, in the exposed mice and their offspring, but not in their grandoffspring (Arai et al. 2009). These effects are transmitted only by females, but persist after cross-fostering. They are mediated by cAMP and p38-dependent signaling, although the underlying epigenetic mechanisms and potential germline alterations remain unknown. As with all studies involving the matriline, maternal effects may be involved and would require cross-fostering to be excluded (see Appendix 1).

Inheritance of Specific Memories While the above findings suggest that cognitive functions can generally be enhanced or impaired, and are likely associated with alterations in signaling networks, a more controversial effect has recently been proposed, suggesting that specific memories formed by the parent generation may be passed to the offspring. A learned association between an odor and a mild footshock was reported in mice to lead to a behavioral sensitization to that odor that can be passed to the offspring and grandoffspring (Dias and Ressler 2014). The inherited memory is odor specific, and the odor can be recognized at lower concentrations (odor sensitization). In the offspring and grandoffspring of odor-conditioned mice, the number of olfactory sensory neurons positive for the M71 receptor, a receptor activated by one of the conditioned odors, acetophenone, and the size of M71-positive glomeruli are increased. These effects can also be transmitted by females and persist after cross-fostering and are even passed from father to offspring by IVF, indeed suggesting transmission through the male germline. A similarly curious phenomenon was noted previously in *C. elegans* larvae, which when exposed to odorants during a critical time window showed increased migration toward the so-called "imprinted" odors, and their offspring but not grandoffspring showed similar increased migration toward the same odor (Remy 2010). If more than four generations of worms each received the same odor imprinting, however, the transgenerational odor preference became extremely persistent and was detectable for at least 40 subsequent generations (Remy 2010). It has to be noted that, although experimentally straightforward, no follow-up studies have been conducted to validate these findings in *C. elegans*. The concept of transmission of a specific odor memory to the offspring is challenging and requires confirmation, as the idea that learning in adulthood can alter epigenetic marks in germ cells remains controversial.

4.3.3 How Do Various Environmental Factors Induce Overlapping Effects in the Offspring?

As described above, different environmental factors including various stressors, drugs of abuse, endocrine disruptors, or different feeding regimes often impact similar behaviors across generations, and several possible explanations for this can be envisaged. Such convergence might be because similar genes are involved. These genes may be located in genomic regions prone to environmental influence that are more susceptible to epimutations. Genomic regions containing nucleosomes are more accessible than regions packaged with protamines in sperm, and certain sequences like CTCF transcription factor binding sites are overrepresented in these regions (Arpanahi et al. 2009). Indeed, undernutrition of embryos *in utero* leads to widespread decreases in DNAme particularly in regions that retain nucleosomes in adult sperm (Radford et al. 2014). Further, various endocrine disruptors were shown to alter DNAme in susceptible genomic regions in sperm cells, and consensus DNA sequence motifs, zinc-finger motifs, and G-quadruplex sequences are overrepresented in these susceptible regions (Guerrero-Bosagna et al. 2010, 2014). But despite these

shared sequences, all endocrine disruptors lead to changes in DNAme in different sets of genes and loci, with minimal to no overlap between them (Manikkam et al. 2012). Thus, it remains unclear how "vulnerable" regions interact with epigenetic modifiers to give rise to epimutations. Alternatively, rather than being more vulnerable, these regions might be more protected from epigenetic reprogramming that occurs following fertilization and germ cell differentiation. Such protection would allow environmentally induced changes to resist epigenetic reprogramming in the early embryo and persist across generations (Wei et al. 2014). Further to DNAme and histone/protamine PTMs, it is also possible that ncRNAs in sperm contribute to non-genetic germline inheritance (Gapp et al. 2014a). By regulating common miRNAs, different environmental factors may target similar downstream gene networks. Consistently, different stress paradigms can regulate distinct but partially overlapping miRNA pools in sperm cells.

It is also possible that different environmental factors cause similar effects because they converge on common signaling pathways and thus regulate shared target genes and behaviors in the offspring. For example, factors like stress, endocrine disruptors, and drugs like alcohol all involve components of glucocorticoid signaling (Haddad 2004; de Kloet et al. 2008; Vitku et al. 2014), and some of these components, in particular glucocorticoid receptors, are expressed in Sertoli cells (Hazra et al. 2014), spermatogonia (Haeussler and Claus 2007), and mature sperm (Kaufmann et al. 1992). In theory, this could allow alterations in glucocorticoid signaling to affect various stages of germ cell development during different developmental stages (embryo, postnatal, adult), possibly with different developmental outcomes. Indeed, repeated injections of the glucocorticoid receptor agonist dexamethasone in adult mice affect DNAme in sperm and alter gene expression and DNAme in different tissues of their offspring (Petropoulos et al. 2014).

A better understanding of the exact mechanisms involved in the induction of non-genetic germline alterations will likely show at which level different environmental factors may converge. Simultaneously, comparing transcriptional profiles of environmental factors that give rise to similar behavioral alterations may reveal common sets of genes that may be located on particularly vulnerable stretches of DNA, or identify shared ncRNA transcripts and targets.

4.4 Possible Mechanisms of Non-genetic Germline Inheritance

The growing evidence in animals and humans that behaviors acquired after an experience or environmental exposure can be heritable and may involve gametes raises several key questions and challenges. First, one of the most important questions to answer is how non-genetic changes can be induced and maintained in germ cells. Then, the time window of induction of these changes needs to be determined. It is not known when environmentally induced changes to the germline can take place and whether there are sensitive periods of induction during germ cell development.

To approach these questions, male germ cells are used as a major point of discussion because up to now, they have been the most directly implicated in non-genetic germline inheritance in mammals. We describe sperm development and epigenetic regulation and propose answers to the questions using examples of demonstrated non-genetic germline inheritance. Although female germ cells also likely contribute to non-genetic inheritance, convincing evidence is currently lacking due to experimental difficulties in working with oocytes (e.g., low number of cells for high-throughput screening) and in circumventing the confounds of intrauterine and maternal factors (see Appendix 1).

4.4.1 Possible Mechanisms of Induction and Maintenance of Non-genetic Marks

Germline non-genetic inheritance requires that marks be induced in germ cells, maintained throughout spermatogenesis, and then transmitted from mature sperm to the zygote at fertilization. The mechanisms involved are likely multiple and may vary depending on the phase of development and generations (parent, offspring, grandoffspring).

4.4.1.1 Induction

It is still unclear how molecular signals induced by environmental factors can reach the germline to elicit non-genetic alterations. Soluble factors in blood are an interesting route via which environmental factors could induce such alterations in sperm. Factors contained in serum from adult rats with chronic hepatic injury can modulate H3K27me3 and H2A.Z at specific genes in sperm of control rats when delivered by serum transfer (Zeybel et al. 2012). Although the nature of these particular factors remains unknown, they seem to originate from rat myofibroblasts differentiated from hepatic stellate cells and can be released in culture medium. These factors can also be produced by human hepatic stellate cells obtained from patients with liver disease when placed in culture. A different study also found that circulating RNAs are factors that can be released from human melanomas and that populate spermatozoa (Cossetti et al. 2014). Circulating RNAs are abundant in blood and are protected from RNases by association with high-/low-density lipoproteins (Vickers et al. 2011) or RNA-binding proteins including Argonaute 2 (Arroyo et al. 2011), or by packaging in exosomes, microvesicles, or apoptotic bodies (Pigati et al. 2010; Pegtel et al. 2010). Exosomes might be able to transport RNAs produced from somatic cells to the germline, by crossing the blood-testes barrier. They could deliver RNAs into germ or Sertoli cells by endocytosis, membrane fusion, or binding to receptors (Zernecke et al. 2009; Vickers et al. 2011; Hergenreider et al. 2012). In mature sperm, endogenous RNA stabilization mechanisms such as those involving MIWI-binding protein may help maintain RNAs in exosomes. In human semen,

exosomes are indeed enriched for miRNAs particularly in seminal plasma (Vojtech et al. 2014). The ability to trace exosomes using specific markers would be useful to determine their cellular origin and destination. Somatic cells in gonads can also influence germ cell epimutations. Sertoli cells and fetal testes express receptors for estrogen and androgen (Majdic et al. 1995; Zhou et al. 2002), which bind to response elements and can recruit DNMTs when activated by estrogenic/androgenic substances (Pathak et al. 2010). Further, there are numerous transporters that allow trafficking between Sertoli cells and postmeiotic spermatids and may provide specific signals to reach the germline (Mital et al. 2011). Peripheral signals released under the control of the CNS such as corticosterone or norepinephrine after stress exposure could also reach germ cells via the blood circulation and bind to their respective receptors on sperm cells (Petropoulos et al. 2014).

Once environmentally induced signals reach the germline, they may be able to alter non-genetic marks possibly by exploiting endogenous mechanisms that normally induce and/or regulate these marks. Different mechanisms might be used depending on the time of induction. Epimutations involving hypomethylation at DMRs during mid-to-late embryogenesis may result from the impaired reacquisition of DNAme during PGC programming. In contrast, hypermethylation of DMRs may result from the use of imprinting-like protection mechanisms such as H3/H4 acetylation (Carr et al. 2007) or trans-acting maintenance factors (Girardot et al. 2013) that prevent reprogramming. These mechanisms may be favored by the proximity to an IAP (see below *Maintenance*). During development, DNAme and histone PTMs can be influenced by stress- or diet-related hormones such as corticosterone, estrogen, leptin, insulin, or by environmental toxins that circulate in blood and/or accumulate in scrotal fat. These factors could activate signaling pathways that can result in the dysregulation of the transcription of DNMTs or histone-modifying enzymes. For example, spermatocytes carry estrogen receptors (ERs) that can influence DNMT1 activity at DMRs (Zhou et al. 2002; Pathak et al. 2010). Estrogens can also regulate chromatin remodeling directly and/or indirectly via follicle-stimulating hormone (Cacciola et al. 2013). Other receptors including TGFbeta1/2 and noradrenergic receptors may also contribute, as they are expressed on different cells during spermatogenesis including spermatogonia, round spermatids, and mature sperm cells and could regulate epigenetic enzymes (Bourguiba et al. 2003; Adeoya-Osiguwa et al. 2006).

Additionally, complex cross-talk between the different non-genetic mechanisms may operate in germ cells and provide a global regulation of non-genetic marks. Changes in DNAme and histone PTMs are able to alter chromatin composition in germ cells. DNAme established early in development seems to prevent nucleosome retention during spermiogenesis, which can result in the enrichment of histones at hypomethylated CpG regions in sperm (Vavouri and Lehner 2011). DMRs involving DNA hypo- or hypermethylation may therefore modulate nucleosome content and chromatin structure. Active H3K4me3 and repressive H3K27me3 not erased during development could also affect nucleosomes in mature sperm and possibly induce divergences in the insertion of histone variants during repackaging of the haploid genome. Cross-talk between DNAme and histone PTMs also exists and

theses epigenetic marks can regulate the expression and the stability of ncRNAs in somatic cells (Hulf et al. 2013; Das et al. 2013). Interactions between epigenetic marks and ncRNAs might occur during different stages of spermatogenesis.

4.4.2 Possible Time Window for the Impact of Environmental Factors on Germ Cells

In mammals, male gametes develop through successive phases that start during embryogenesis and continue throughout postnatal and adult life. Each phase can potentially be affected by experiences or environmental exposure, but prenatal and early postnatal phases are particularly vulnerable because they correspond to the time when epigenetic marks are established. Before they are fully established and stabilized, these marks are still malleable. But once fixed, they are more resistant to external influence. The time window of exposure thus determines the type and degree of cellular and molecular targets that are affected.

4.4.2.1 Development of Male Germ Cells in Rodents

Germ cells start to develop in the fetus shortly after fertilization when a population of pluripotent cells gives rise to primordial germ cell (PGC) precursors in the epiblast (between E3.5 and E6). PGCs emerge from the epiblast at E7.25 as a cluster of about 20 cells that settle in the fetal gonads. These cells rapidly proliferate and migrate to the genital ridge. Sex determination in these cells occurs at E12.5. Once carrying sex specificity, PGCs stop dividing at E13.5 and give rise to mitotically arrested prospermatogonia (gonocytes). The first wave of spermatogenesis starts shortly after birth in seminiferous tubules in the testis and lasts about 35 days in rodents. Seminiferous tubules are composed of Sertoli cells that divide the tubules into basal and apical compartments and tightly control spermatogenesis (Rossi and Dolci 2013). Prospermatogonia first develop into spermatogonial stem cells (SSCs), self-renewing cells maintained across life. SSCs proliferate by consecutive mitotic divisions and mature into spermatocytes during the first postnatal week. Spermatocytes then enter meiosis until around PND19 (Nebel et al. 1961). During preleptotene meiosis, they cross the blood-testis barrier that starts to be established by Sertoli cells in the testis (Mital et al. 2011). In rodents, the blood-testis barrier is functional around PND20 (Vitale et al. 1973). Testis-specific histone variants start to be installed around preleptotene. Round spermatids, the earliest postmeiotic cells, are produced around weaning (PND20–22). They turn into elongating spermatids after about 2–3 weeks, during which most histones (90–95 % in human, 95–99 % in mouse) are replaced by transition proteins, then by protamines for chromatin compaction. Spermatids mature into sperm cells that are released in the lumen of the seminiferous tubules starting around PND35 in mice (PND44 in rat). SSCs self-renewal and spermatogenic development are recapitulated in the testis

throughout adulthood. Spermatogenesis requires about 40 days in mice (Hess and Renato de Franca 2008) and 65 days in rats (Hermo et al. 2010). Multiple spermatogenesis cycles occur simultaneously and sequentially. Spermatogenesis is dynamically controlled by hormones, i.e., testosterone and gonadotropins, and developing germ cells express various receptors at different stages of maturation. While late spermatocytes and round spermatids are transcriptionally active, elongating spermatids and sperm cells are inactive. Developing germ cells also contain cytoplasmic RNA granules including P- and chromatoid bodies present throughout spermatogenesis. The composition of these granules remains largely unknown, but includes RNAs and proteins critical for sperm development (Meikar et al. 2011; Nguyen-Chi and Morello 2011).

4.4.2.2 Environmental Impact During Embryogenesis

Epigenetic marks in germ cells are strongly influenced by environmental factors in prenatal life. E8–E14 exposure to endocrine disruptors and toxicants like vinclozolin, plastics, dioxin, jet fuels, or alcohol (E7–21) triggers epimutations in differentially methylated regions (DMRs) in sperm (Manikkam et al. 2012; Skinner et al. 2013), indicating that fetal germ cells during reprogramming are sensitive to multiple external factors. Embryonic DMRs can be perpetuated across generations. DMRs induced by E8–E14 vinclozolin treatment persist in promoter regions in PGCs, prospermatogonia, and mature sperm in grandoffspring (Guerrero-Bosagna et al. 2010). Transmission likely depends on early and/or extended treatment, since vinclozolin treatment at E15–E21 has no effect on F1 testis (Uzumcu et al. 2004). Undernutrition by caloric restriction during the last week of gestation (from E12.5 to birth) in mice leads to widespread epimutations in adult sperm (Martínez et al. 2014; Radford et al. 2014). Some of these epimutations are still detected in the liver of the progeny (Martínez et al. 2014) and lead to changes in gene expression and metabolic dysfunctions. However, protein restriction after weaning does not induce epimutations in adult sperm, suggesting germ cells later in life are less susceptible to perturbations (Carone et al. 2010). In all cases of *in utero* exposure, if PGCs are affected, the resulting gametes produced later in life likely carry a memory of the exposure that can be maintained and transmitted to subsequent generations.

4.4.2.3 Environmental Impact During Early Postnatal Life

Postnatal germ cells remain sensitive to the environment, in particular early after birth. In humans, the prepuberty slow-growth period (before age 15) is highly vulnerable to changes in food supply with durable effects across generations (Kaati et al. 2007). In mice, unpredictable traumatic stress from postnatal day (PND) 1–14 induces epimutations in sperm in the exposed mice and their progeny, which are accompanied by behavioral effects across three generations (Franklin et al. 2010). It also alters ncRNAs in sperm, brain, and serum in adulthood (Gapp et al. 2014a). High-fat diet from weaning to 9–12 weeks or later also alters

DNAme in sperm, i.e., at metabolic genes, and these effects persist across three generations (Fullston et al. 2013; Wei et al. 2014). The sensitivity of postnatal germ cells may result from their responsiveness to signaling molecules like hormones, cytokines, or growth factors possibly released locally (e.g., Sertoli cells) or systemically in the blood circulation, for instance, upon environmental exposure. Receptors for these molecules are expressed at early stages of each spermatogenic cycle (Naz and Sellamuthu 2006). Around PND20, the blood-testis barrier established by Sertoli cells provides a protection of germ cells from external factors. However, developing and possibly also adult Sertoli cells are themselves vulnerable, and their epigenome and transcriptome can be altered by environmental exposure during early embryogenesis, for instance, by vinclozolin, with effects up to the grandoffspring in rat (Guerrero-Bosagna et al. 2013). Since fully differentiated Sertoli cells no longer proliferate, if they are impaired in their functions or biology, this may have an impact on sperm cell development and integrity throughout adult life.

4.4.2.4 Environmental Impact During Adulthood

Since spermatogenesis continues throughout life, the adult individual still carries immature germ cells that remain susceptible to environmental insult. In the adult, these cells may be differently affected depending on their stage of development when exposure occurs, and on the duration of the exposure. They are also subjected to the natural epigenomic variability that undergoes in developing germ cells during spermatogenesis (Marques et al. 2011). Exposure spanning one or more spermatogenic cycles may affect cells at all stages, but if spermatogonia are not affected, cells from the following cycles will be normal. If spermatogonia are affected, cells from all stages and all spermatogenesis cycles will carry the perturbations. Once mature sperm cells leave the germinal epithelium where they are protected, they may become more vulnerable to environmental factors, although the blood-epididymis barrier can also provide protection (Aitken et al. 2004; Mital et al. 2011). Exposure to chemicals in adulthood has been reported to induce non-genetic alterations in sperm cells. Eight-day methoxychlor treatment in adult mice affects DNAme at several imprinted genes in sperm (Stouder and Paoloni-Giacobino 2011). Although transmission has not been tested in this model, such epimutations have been observed in the F1 and F2 offspring of females with a similar treatment of 8 days between E10 and E18, suggesting continued sensitivity of imprinted loci. A 4-week treatment with a hepatoxin or 8-week administration of cocaine modifies histone composition and PTMs in sperm, respectively (Zeybel et al. 2012; Vassoler et al. 2013). Further, chronic paternal stress from adolescence to adulthood also alters sperm miRNAs, but is not associated with any behavioral alterations (Rodgers et al. 2013). A 10-week high-fat diet initiated during adolescence (5 weeks postnatal) in mice induces global hypomethylation in testis and spermatids and alters miRNA expression in testis and sperm. Such treatment has metabolic consequences across two generations (Fullston et al. 2013). Longer exposure (16-week) however triggers sperm DNA damage in part because it affects the expression of SIRT6, a histone deacetylase involved in DNA repair, suggesting impaired gametic integrity

(Palmer et al. 2011). Exposure later in life may even have more dramatic consequences, since the susceptibility of the sperm epigenome to environmental insults seems to increase with advanced paternal age (Rando and Chang 2012).

4.4.3 Possible Mechanisms of Maintenance of Non-genetic Changes in Germ Cells

Non-genetic marks acquired in the germline upon environmental exposure need to be maintained until transferred to the zygote during fertilization. When acquired during embryogenesis or early postnatal life, marks involving DNAme and histone PTMs have to resist the waves of epigenetic reprogramming (Jenkins and Carrell 2012; Seisenberger et al. 2013). To be maintained, epimutations may use mechanisms of maintenance that normally operate during reprogramming. They may involve DNA-binding proteins like MBD3, ZFP57, KAP1, ARID4A-B, or CTCF or DNMT-targeting proteins such as NP95 (Uhrf1) (Bostick et al. 2007; Sharif et al. 2007) and specific sequence motifs, i.e., CTG/CAG repeats, which can favor or prevent DNA accessibility to DNMTs and associated enzymes (Bartolomei 2009; Girardot et al. 2013). Protective proteins like PGC7/Stella/DPPA3 can preserve 5mC from TET3-dependent oxidative demethylation by binding to H3K9me2 (Nakamura et al. 2012). DNAme can also be preserved by Piwi proteins MILI/MIWI2, which interact with Piwi-interacting small RNAs (piRNAs) that help maintain transposon silencing (Carmell et al. 2007; Aravin et al. 2007). Conversely, TET1/2, which erases imprinted DNAme in PGCs by conversion of 5mC into 5hmC, might also be recruited (Hackett et al. 2013). Altered ncRNA composition could possibly be maintained by epigenetic regulation of the genes coding for the ncRNAs through DNAme and/or histone PTMs. Once produced, ncRNAs could be stabilized by methylation through RNA methyltransferases such as DNMT2 (Kiani et al. 2013). But ncRNA alterations might also be relayed by other non-genetic mechanisms in the offspring, which are currently not known. Therefore, ncRNAs may only represent initial triggers of non-genetic modifications that have the ability to perpetuate these modifications (Gapp et al. 2014a). Indeed since DNAme, histone/protamine modifications, and ncRNAs can efficiently cross-talk to regulate the genome and its activity, together, they may contribute to maintain non-genetic changes.

4.5 Outlook and Conclusions

In the past couple of years, solid evidence for germline-dependent non-genetic inheritance of traits induced by the environment in mammals has accumulated in the fields of environmental toxicology, neuroscience, behavioral neuroendocrinology, and nutritional science (Bohacek and Mansuy 2015; Nilsson and

Skinner 2014). This mode of inheritance is critical for medical genetics because it helps explain the etiology, expression, and heritability of prevalent metabolic or neuropsychiatric diseases, including diabetes, depression, anxiety, and personality disorders or autism (Manolio et al. 2009; Bohacek and Mansuy 2013). Identifying non-genetic marks and mechanisms may provide biomarkers for disease diagnosis and treatment monitoring, possibly also for prevention (Sharma 2012). Drugs correcting epigenetic defects such as DNMTs or HDAC inhibitors may be useful, but since epigenetic marks are often altered bidirectionally (e.g., DNA hypo- and hypermethylation), their action can only be limited. Further, some of these drugs have recently been reported to alter the epigenome in germ cells (Jia et al. 2014), which may lead to uncontrollable, nonspecific, and long-term alterations.

Non-genetic germline inheritance may also have a role in evolution. It could represent a means to rapidly transfer relevant environmental information to the offspring. In some cases, it may provide an adaptive advantage in dynamic environments by broadening the panel of behavioral responses. Epimutations may also introduce genetic variability by favoring DNA recombination or transposition (Boyko et al. 2007) and alter genomic stability as observed in cancer (Feinberg et al. 2006). In outbred populations, there is likely a complex interplay between genetic and epigenetic changes, for example, domestication elicits pronounced differences in DNAme and non-Mendelian patterns of inheritance (Markel and Trut 2011; Nätt et al. 2012). Epimutations can also affect mate selection and impact natural selection (Crews et al. 2007). Non-genetic germline inheritance therefore needs to be integrated into the modern view of evolution (Jablonka 2012), even if its underlying bases remain not fully understood. Studies on ancient tissues or recently evolved species should help gain insight into the evolutionary timescale and correlation with the genome (Llamas et al. 2012; Skinner et al. 2014).

Studies of non-genetic germline inheritance have several major challenges ahead. They require that reported cases of non-genetic inheritance provide solid causal evidence for the implication of germ cells. Because the impact of this concept is so tremendous, high standards are required: extraordinary findings need extraordinary evidence. Several experimental elements are thus essential, such as the confirmation of inheritance in independent cohorts of animals, the assessment of epigenetic marks in sperm cells across generations and during development, and the exclusion of non-germline factors such as maternal behaviors through the use of assisted reproductive techniques or innovative approaches like injection of sperm RNAs into fertilized oocytes (see Appendix 1). Methodological advances in next-generation sequencing (Chip-Seq, RNA-Seq, single-cell sequencing) in sperm cells and oocytes should help gain knowledge on non-genetic marks involved in inheritance, identifying possible epimutation hotspots and features of susceptible regions in germ cells. Less common non-genetic marks like hydroxymethylation, non-CpG methylation, and long ncRNAs, piRNAs, or protamine PTMs should also receive attention.

Appendix 1: Experimental Methods to Examine Different Routes of Non-genetic Inheritance of Acquired Information in Rodents

Acquired information can be transmitted to the offspring via several routes that do not involve germ cells, and these factors can operate during mating, *in utero*, or peri- or postnatally in mammals. (1) During mating, seminal fluid is transmitted from male to female in addition to sperm. The composition of seminal fluid can change in response to environmental factors and influence the offspring independently from sperm (Perry et al. 2013). In flies, it was shown that seminal fluid can transfer acquired features from a male which mated but did not fertilize the female, to the offspring sired by a subsequent male (Crean et al. 2014). (2) In mammals, *in utero* components such as hormones, immune factors, nutrients, or toxins can influence the fetus and elicit persistent phenotypic traits later in life. Stress in gestating females can alter the offspring's emotional, social, and cognitive abilities (Weinstock 2008; Moisiadis and Matthews 2014), in part by altering plasma glucocorticoids or androgens. Exposure to specific flavors or odors can also modulate the olfactory system and taste preference in the offspring (Schaal et al. 2000; Ong and Muhlhausler 2011; Todrank et al. 2011). In certain bird species, females can load antibodies against encountered pathogens into the eggs' yolk, providing protection to their progeny (Frésard et al. 2013). (3) Peri- and postnatally, the amount and quality of maternal care strongly influence the offspring's behavior in adulthood. Female pups receiving poor maternal care will also show reduced maternal behaviors themselves, which is associated with epigenetic dysregulation in the brain (Weaver et al. 2004; Champagne 2008). Additionally, milk composition during lactation (Liu et al. 2014), microbiota (Stilling et al. 2014), or odor cues (Debiec and Sullivan 2014) can also transfer environmental experiences from mother to offspring.

Distinguishing these germline-independent forms of inheritance from non-genetic germline-dependent components is challenging and requires specific experimental strategies. Using patrilines helps control the influence of some *in utero* and postnatal factors, but the presence of the male during mating may still exert an influence on the embryo. Additionally, rodent females have been reported to adjust maternal investment based on the sire's fitness (Curley et al. 2011; Mashoodh et al. 2012). The following strategies can be used to control some of these confounding factors: (1) Cross-fostering of pups to a control dam can be used to exclude the contribution of peri-/postnatal maternal influences (Bohacek et al. 2015). (2) Artificial insemination or *in vitro* fertilization (IVF) can directly test transmission through the germline and avoid confounding effects of seminal fluid and interactions during mating (Dietz et al. 2011; Dias and Ressler 2014). IVF – routinely available in many facilities – involves *in vitro* culture conditions which, in addition to superovulation, can create further uncontrollable epigenetic confounds (Denomme and Mann 2012). Artificial insemination has rarely been used in mice, but new simplified protocols are now available and should be used more routinely in studies of non-genetic germline inheritance (Bohacek et al. 2016). (3) Embryo transfer can

be particularly useful in female line studies to avoid *in utero* and postnatal maternal effects, but also involves *in vitro* biases. (4) Direct injection of molecules such as sperm RNAs into fertilized wild-type oocytes followed by transplantation in a pseudo-pregnant mother is another elegant approach to directly test the contribution of specific germline components (Rassoulzadegan et al. 2006; Gapp et al. 2014a). These procedures can help determine the germline dependence of non-genetic inheritance and should be employed more systematically but with caution since some can affect epigenetic reprogramming.

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Chapter 5
Epigenetic Alterations to *NR3C1* and *HSD11B2* and the Developmental Origins of Mental Disease Risk

Allison A. Appleton, Elizabeth A. Holdsworth, and Mary Elizabeth Ingle

Abstract Gestation is one of the most critical periods of human development where adverse exposures occurring during pregnancy can shape the health of the offspring over the life course and contribute to the intergenerational transmission of disease risk. Many psychiatric conditions are now thought to originate in part *in utero*. As such, a myriad of gestational exposures may contribute to the biological embedding of poor mental health for the next generation. Increasingly, researchers are examining epigenetic alterations as mechanisms linking prenatal exposures to offspring mental disease risk. Epigenetic alterations, which can functionally regulate gene expression and thus phenotype, are tremendously sensitive to intrauterine exposures. Epigenetic mechanisms related to neuroendocrine regulation may be the linking mechanisms between adverse in utero exposures and later life poor mental health. This chapter reviews the evidence linking (1) prenatal exposures to epigenetic modification of genes involved in the regulation of cortisol (*NR3C1* and *HSD11B2*) and (2) how such epigenetic alterations can in turn lead to changes in offspring mental disease risk.

Keywords Epigenetics • HPA axis • *NR3C1* • *HSD11B2* • Developmental origins of health and disease

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5.1 Introduction

In order to better understand the etiology of mental conditions and identify novel avenues for prevention and treatment, examining the intergenerational transmission of mental health conditions has become of increasing interest to researchers and clinicians alike (Schlotz and Phillips 2009). Many mental and behavioral health conditions are now thought to be attributable in part to an adverse intrauterine environment (Barker 1998; Schlotz and Phillips 2009; Raikkonen et al. 2012; Sandman and Davis 2012), with epigenetic alterations to the developing fetus' hypothalamic-pituitary-adrenal (HPA) axis emerging as important explanatory mechanisms linking gestational exposures to later life disease risk among the offspring. Working from a Developmental Origins of Health and Disease framework and an epidemiologic perspective, this chapter reviews (1) the evidence linking prenatal exposures to gestational epigenetic alterations to two HPA-axis gene promoters involved in the regulation of cortisol (*NR3C1*, *HSD11B2*) and also (2) the evidence linking such in utero epigenetic changes to postnatal neuroendocrine and neurobehavioral outcomes. In this review, we emphasize findings from prospective human studies that examine DNA methylation in cord blood or placenta tissue, thereby capturing the epigenetic signatures attributable to prenatal exposures and not the postnatal environment. Following the review, we identify gaps in the evidence base and suggest opportunities to pursue to move the field forward. This burgeoning area of research holds tremendous promise for elucidating the molecular mechanisms involved in the early origins of mental disease risk. We begin our review of the evidence with a brief orientation to the Developmental Origins of Health and Disease approach and its relevance and application to epigenetic studies of the intergenerational transmission of mental disease risk.

5.2 The Developmental Origins of Health and Disease Framework

The Developmental Origins and Health and Disease (DOHaD) framework specifies that exposures occurring during critical periods of development like gestation can influence health risk over the life course in the offspring and potentially across generations (Barker 1998; Ben-Shlomo and Kuh 2002). Also known as the "fetal origins" or "Barker hypothesis," this framework was initially developed from a series of epidemiologic studies demonstrating that measures of birth size were associated with increased risk of developing cardiovascular diseases in midlife, including coronary heart disease, hypertension, type II diabetes, and stroke (Barker 1998). It was hypothesized that fetal undernutrition, indexed by being born with low birth weight, permanently altered the structure and function of the developing fetus' cardiovascular system and metabolic processes. This in utero "programming" in turn altered one's risk of developing cardiovascular disease over the life course. In the 25 years

since the DOHaD framework was first proposed, it has become generally accepted that birth size is a marker for many aspects of the intrauterine environment in addition to nutritional influences and that birth weight in particular is a proxy indicator for a complex interplay of underlying etiologic mechanisms affecting both intrauterine growth and adult physiological systems. This approach has highlighted the tremendous sensitivity of the gestational period for modulating disease risk for a lifetime and has become a burgeoning area of study.

Evidence is accumulating that the origins of many adulthood chronic diseases, including mental health conditions like depression and schizophrenia, can be traced to exposures acting during the earliest times in life (Buka et al. 2001; Cannon and Rosso 2002; Gale and Martyn 2004; Alati et al. 2007; Banerjee et al. 2007). Moreover, current DOHaD research focuses on a wide range of intrauterine exposures including psychosocial stress, environmental toxicants, smoking, and other lifestyle factors in addition to prenatal nutrition. The field has also moved beyond birth weight as a marker for deleterious intrauterine exposure and disease risk. For example, an exciting body of work has linked a range of prenatal exposures to infant neurodevelopment and neuroendocrine function (Lester et al. 2011, 2013, 2014). Moreover, the molecular and epigenetic mechanisms linking gestational exposures to later disease risk have become increasingly appreciated and are the focus of much recent DOHaD work (Meaney and Szyf 2005; Monk et al. 2012). In this regard, the DOHaD framework can contribute important insights into understanding how a range of exposures during gestation can become biologically embedded to affect the development of the neuroendocrine system and mental health risk, both within and across generations. Thus, this chapter focuses on the gestational determinants of neurodevelopment and risk of mental disease and the epigenetic mechanisms that help explain such associations.

5.3 DOHaD, HPA-Axis Development, and Epigenetic Modulation

Many of the acquired adverse effects related to intrauterine environment result in epigenetic alterations. Epigenetics involves alterations to an individual's genome without changing the underlying DNA nucleotide sequence. The most widely studied epigenetic alteration is DNA methylation, which occurs with the addition of a methyl group to the 5′-carbon of the cytosine in CpG dinucleotides and in the context of multiple methylated cytosines within a particular gene regulatory region. Such methylation is often associated with total transcriptional silencing of the downstream gene. The presence of the methyl group alone in this context is not sufficient for transcriptional silencing, but instead recruits component proteins related to gene repression. This results in creation and maintenance of a silenced closed/compacted chromatin conformation. Thus, the presence of the methylation changes is indicative of these alterations. Epigenetic mechanisms are particularly relevant for understanding the developmental origins of disease as they are sensitive to a

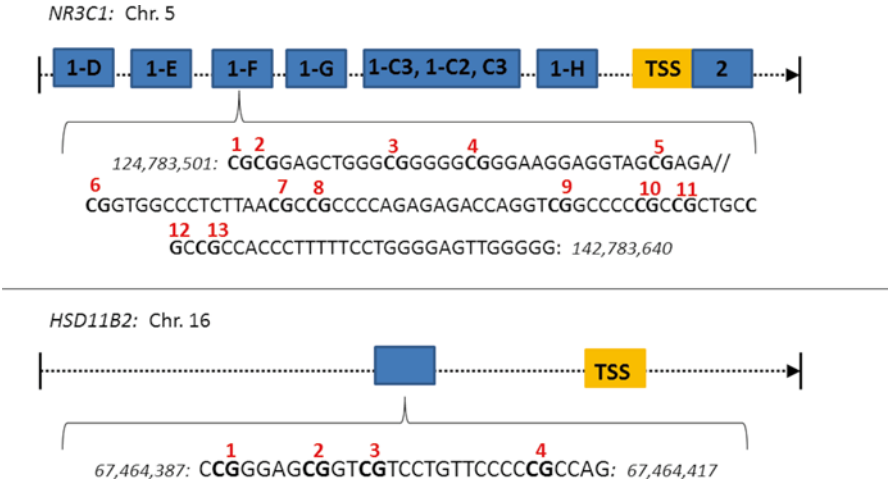

Fig. 5.1 Regions of interest for *NR3C1* and *HSD11B2*. Transcriptional start site shown in *orange*; exons are represented in *blue*

wide range of exposures and can be altered during critical periods of development like gestation, and such alterations can remain stable across the life course.

The neurotoxic effects of deleterious prenatal exposures may operate through epigenetic alterations to the hypothalamic-pituitary-adrenal (HPA) axis. This systemic pathway involves neuroendocrine signaling between the hypothalamus and pituitary glands in the brain and the adrenal glands and regulates the body's response to stress and a range of biological and physiologic processes. Dysregulation of the HPA axis is linked to disease risk for several mental and physical health conditions. The HPA axis is programmed during fetal development to "hard wire" fetal physiological systems for optimal adaptation in the postnatal environment. Disruption of HPA development, particularly through epigenetic alterations to genes involved in the HPA axis, may effectively program and dysregulate the body's stress response system, thus contributing to poor neurodevelopment and mental disease risk over the life course.

Recent work suggests gestational HPA-related epigenetic alterations of genes involved in the HPA axis affect neurodevelopment, and such epigenetic alterations are sensitive to a range of prenatal exposures (Lester et al. 2014). Work in this area is growing rapidly; epigenetic alterations to a number of HPA-related genes seem to be sensitive to prenatal exposures, with significant neuroendocrine sequelae (Lesseur et al. 2013, 2014a, b, c; Paquette et al. 2013, 2014). The evidence is most well established for genes involved in the regulation of cortisol including the glucocorticoid receptor (NR3C1) and 11-β hydroxysteroid dehydrogenase type 2 (HSD11B2), which are the focus of this review (see Fig. 5.1 for a schematic of gene structure and regions of interest for NR3C1 and HSD11B2). NR3C1 is the nuclear receptor to which glucocorticoids like cortisol bind: cortisol circulates in the bloodstream, binds to these receptors, and, in so doing, reduces HPA activity and

further cortisol secretion. Methylation of *NR3C1* effectively silences the expression of the gene, which reduces the number of receptors available for cortisol to bind to, thus potentially resulting in larger degrees of circulating cortisol levels in the blood. Conversely, HSD11B2 is a protective mechanism that prevents glucocorticoid overexposure. HSD11B2 converts cortisol into inactive cortisone, thereby protecting the fetus from overexposure to harmful stress hormones during development. Dysregulation of NR3C1 and HSD11B2 has been linked to risky perinatal outcomes, like being born with low birth weight (Bromer et al. 2012; Marsit et al. 2012; Zhao et al. 2014) and poor neurodevelopment (Lester et al. 2014) and also major mental health conditions, including depression (Januar et al. 2015), posttraumatic stress disorder (Zannas et al. 2015), anxiety, and cardiometabolic disorders (Cottrell and Seckl 2009; Wyrwoll et al. 2011). In the next sections of this chapter, we review the extant literature to characterize the gestational exposures that contribute to epigenetic alterations to *NR3C1* and *HSD11B2* and to the neurodevelopmental and neuroendocrine outcomes of such gestational epigenetic changes that in turn set the state for mental and behavioral health risk for the next generation.

5.4 Gestational Exposures and Epigenetic Alterations to Offspring *HSD11B2* and *NR3C1*

In a rapidly growing area of research, epigenetic alterations to offspring *NR3C1* and *HSD11B2* have been found to associate with several different exposures from prenatal life. Because of the hypersecretion of cortisol that accompanies depression, stress, and other emotional states, most of the research in this area has focused on evaluating whether prenatal exposure to such psychosocial factors programs the offspring's own HPA axis, through epigenetic modulation of its cortisol regulation pathway, indexed by methylation to offspring *NR3C1* and *HSD11B2*. Other emerging work has considered prenatal exposure to low socioeconomic status, smoking, preeclampsia, and some environmental toxicants as also modulating *NR3C1* and *HSD11B2* methylation during gestation. We now turn to reviewing notable studies in these areas. These studies are also summarized in Table 5.1.

5.4.1 Prenatal Exposure to Stress, Depression, and Trauma

The intergenerational epigenetic effects of prenatal stressors, including depression and anxiety, warrant research given the prevalence of these conditions. A recent review found that prevalence rates of depression during pregnancy were 7.4 %, 12.8 %, and 12.0 % for the first, second, and third trimesters respectively (Bennett et al. 2004). Another study found even higher rates, with 54 % of pregnant women experiencing antenatal anxiety during any point in pregnancy and 37.1 % experiencing antenatal depressive symptoms (Lee et al. 2007).

Table 5.1 Studies examining association between gestational exposures with NR3C1 and/or HSD11B2 methylation

Citation	Population	n	Gestational exposure	Epigenetic outcome
Appleton et al. (2013)	Healthy term pregnancies, Providence, Rhode Island, USA (RICHS)	444	Socioeconomic adversity	Decreased placental HSD11B2 methylation; effects stronger for male infants
Conradt et al. (2013)	Healthy term pregnancies, Providence, Rhode Island, USA (RICHS)	521	Anxiety and depression	Increased placental *HSD11B2* and *NR3C1* methylation
Goodrich et al. (2015)	Cohort of healthy mother-infant pairs in Mexico City (ELEMENT)	247	Lead	Increased *HSD11B2* methylation in infants
Hogg et al. (2013)	Women with preeclampsia and healthy controls in Vancouver, Canada	148	Preeclampsia	Increased placental *NR3C1* methylation; no association with *HSD11B2*
Hompes et al. (2013)	Population-based study of pregnancy stress in Belgium	83	Anxiety and depression	Increased *NR3C1* cord blood methylation
Hu et al. (2014)	Women with preeclampsia and healthy controls in Hangzhou, Zhejiang, China	121	Preeclampsia	Decreased *HSD11B2* cord blood methylation
Mulligan et al. (2012)	War survivors from the Democratic Republic of Congo	25	Extreme war-related stress, financial stress, and everyday stressors	Increased *NR3C1* cord blood methylation, lower infant birth weight
Oberlander et al. (2008)	Women with depression and healthy controls from Vancouver, Canada	82	Depression	Increased *NR3C1* cord blood methylation
Perroud et al. (2014)	Tutsi genocide survivors and controls	50	PTSD and war trauma	Increased *NR3C1* methylation in peripheral blood among teenaged offspring
Stroud et al. (2014)	Healthy mothers from a low-income, racially/ethnically diverse sample in Providence, Rhode Island, USA	45	Cigarette smoking	Increased *NR3C1* cord blood methylation
Yehuda et al. (2014)	Offspring of Holocaust survivors	95	Parental PTSD	Increased *NR3C1* methylation in peripheral blood among adult offspring

The first human study to establish the association of prenatal exposure to maternal depression, gestational methylation of *NR3C1*, and infant neuroendocrine function was Oberlander and colleagues (Oberlander et al. 2008). They conducted a prospective study of 82 pregnant women (46 were depressed, 36 were not depressed) and examined whether trimester-specific measures of prenatal depression were associated with methylation of *NR3C1* in cord blood at birth and whether such methylation was associated with infant cortisol response to a laboratory-based stressor at 3 months of age. Maternal depression was assessed with validated instruments, including the Hamilton Depression Scale and the Edinburgh Postnatal Depression Scale. The authors found that increased second and third trimester depressive symptoms were associated with increased methylation extents of CpG sites 1–3 of exon 1F of *NR3C1,* controlling for prenatal exposure to serotonin reuptake inhibitor antidepressant use, gestational age at birth, and APGAR scores. *NR3C1* CpG3 methylation was in turn associated with infant cortisol reactivity at 3 months of age (Oberlander et al. 2008). This prospective study provided the first evidence in humans for an intergenerational link between maternal depressed mood and infant neuroendocrine function, by way of epigenetic modulation of *NR3C1*, likely via overexposure to glucocorticoids during fetal development. Several studies have since detected a transgenerational epigenetic effect of prenatal exposure to depression, including one recent study among 57 infants where maternal prenatal depression was associated with *NR3C1* methylation among male infants at 2 months of age (Braithwaite et al. 2015).

Since the publication of the Oberlander study, research on prenatal depression and psychosocial stress and epigenetic alterations to the offspring's cortisol regulation pathway have increased in number as well as expanded the scope of exposure assessment. One study of pregnant women from Belgium ($n = 83$) extensively characterized prenatal HPA activity, psychosocial stress, and emotional health in association with *NR3C1* methylation at birth (Hompes et al. 2013). Specifically, Hompes and colleagues examined trimester-specific maternal cortisol levels alongside multiple validated questionnaire-based psychosocial stress and emotional health assessments, including the Pregnancy-Related Anxiety Questionnaire (e.g., fear of giving birth, fear for the integrity of the baby, fear for changes to one's appearance), the State-Trait Anxiety Inventory, and the Edinburg Depression Scale, in association with cord blood *NR3C1* methylation at various regions. Univariate associations were noted across multiple stress markers and time points, with findings most robust for pregnancy-related anxiety and *NR3C1* methylation at exon 1F CpG9 (Hompes et al. 2013).

These findings are congruent with other studies focusing on more extreme forms of prenatal stress and poor mental health, including exposure to war-related trauma and posttraumatic stress disorder. For example, a recent study of 25 pregnant women from the Democratic Republic of Congo examined financial stressors (material deprivation), everyday stressors (e.g., do you have help at home?), and extreme stress (e.g., war-related experience or witness to rape, execution, and kidnapping) in association with cord blood *NR3C1* methylation and infant birth weight (Mulligan et al. 2012). Correlations were found with each form of stress, *NR3C1* methylation,

and infant birth weight, with associations most robust for the associations between extreme war-related stressors. Findings were similar in another study of war trauma and posttraumatic stress disorder (PTSD) and transgenerational epigenetic effects to *NR3C1* methylation among teenage children born to Tutsi widows pregnant during the genocide ($n=25$) as compared to children born to Tutsi women not exposed to the genocide during pregnancy ($n=25$) (Perroud et al. 2014). Indeed, a growing body of work indicates that PTSD may be responsible for modulating the transgenerational epigenetic effects to *NR3C1* in such cases of traumatic stress. For example, Yehuda and colleagues studied *NR3C1* methylation in adults with at least one Holocaust survivor parent ($n=80$) and demographically matched controls ($n=15$); differential DNA methylation patterns emerged according to paternal and maternal PTSD (Yehuda et al. 2014). We refer the reader to an extensive discussion of the role of PTSD in the transgenerational transmission of epigenetic modulation to *NR3C1* in this volume (see Bowers et al. Chap. 6).

By far, most of the work examining prenatal exposure to stressors and poor mental health in association with offspring methylation to HPA-related genes has focused on *NR3C1*. While fewer in number, there are a congruent set of animal and human studies that highlight linkages between prenatal psychosocial stress, maternal mental health, and epigenetic alterations to *HSD11B2* among offspring. For example, one study of rats exposed to a chronic stressor (forced restraint; $n=6$) during gestational days 14–20 found differential patterns of *HSD11B2* methylation across fetal tissues and stress conditions (Peña et al. 2012). Stressed animals exhibited significantly higher *HSD11B2* placental methylation (CpG sites 4, 5, 7, 8, and 15) and fetal hypothalamus methylation (CpG sites 21 and 22) compared to non-stressed controls. Significantly lower levels of methylation were also observed in fetal hypothalamus tissue at other *HSD11B2* promoter locations (CpG sites 2, 3, 4, 6, 7, 8, 15, and 33). Though small, this study illustrates the sensitivity of *HSD11B2* to prenatal stress and also underscores the variability of epigenetic signatures across tissues (Peña et al. 2012).

There have been few prospective human studies of any prenatal exposure with *HSD11B2* methylation among offspring. Two studies that have examined prenatal psychosocial exposures with placental epigenetic alterations come from a large population-based cohort of healthy term pregnancies (the Rhode Island Child Health Study; described at length in the next section of this chapter). In this cohort, Conradt et al. (2013) found that infants whose mothers had anxiety during pregnancy had greater placental methylation of *HSD11B2* (GpG 4) as compared to those without anxiety; similarly, infants born to mothers with depression had greater *NR3C1* methylation (CpG 2) than infants born to non-depressed mothers. These epigenetic alterations in turn associated with worse neonatal neurobehavior. Also in this cohort, we found that low socioeconomic status during pregnancy (a marker of chronic psychosocial stress, measured by several factors including low education attainment, living in poverty, and living in an overcrowded dwelling) was associated with placental methylation of *HSD11B2,* particularly among the male infants (Appleton et al. 2013). Interestingly, this study found greater maternal socioeconomic adversity to associate with less placental methylation of *HSD11B2*. Such patterning

was consistent with developmental origins of disease framework whereby environmental cues transmitted from the mother during gestation may have programmed the developing fetus's response to an adverse postnatal environment. Less methylation of placental *HSD11B2* may therefore be adaptive and promote the effective management of stress associated with social adversity. Future work should build on these studies to continue to characterize the relation between prenatal stress and mental health exposures with *HSD11B2* epigenetic alterations among offspring.

5.4.2 Other Exposures

In addition to maternal stress and mental health, a varied range of other gestational exposures appear to modulate the developing cortisol regulation pathway for the next generation. These associations include preeclampsia, prenatal smoking, and metals exposure during pregnancy. For example, emerging research concerning preeclampsia, a pregnancy condition characterized by hypertension that affects approximately 4 % US pregnancies (Ananth et al. 2013), finds that this condition affects offspring *HSD11B2* and *NR3C1* methylation. Preeclampsia reduces placental expression and activity of HSD11B2, thus contributing to an increase in fetal exposure to glucocorticoids during development. In two separate studies comparing preeclamptic women with normotensive pregnancies, one study found preeclampsia to be associated with hypomethylation of cord blood *HSD11B2* (Hu et al. 2014); the other study found preeclampsia to be positively associated with placental *NR3C1* methylation but not *HSD11B2* (Hogg et al. 2013).

Similarly, while cigarette smoking during pregnancy has been consistently linked to poor perinatal outcomes and adverse child and adolescent behavior problems (Banerjee et al. 2007; van den Burg et al. 2012), only one study has specifically examined whether offspring *NR3C1* methylation can help explain how smoking risks gets transmitted across generations to affect offspring mental health. This study included 100 healthy mother and infant pairs, where smoking during pregnancy was biologically verified and found to contribute to dysregulated cortisol reactivity in response to a stressor among infants at 5 months; an analysis among 45 of the participants suggested that *NR3C1* cord blood methylation mediated the association (Stroud et al. 2014). These findings are congruent with other association studies that have found that prenatal tobacco smoke exposure modulates other regions of the child's epigenome (Breton et al. 2014).

Finally, emerging evidence suggests that metals exposure during pregnancy may play a role in modulating HPA-related epigenetic alterations. Metals like lead and mercury are known neurotoxicants and have been linked to mental, behavioral, and cognitive deficits (Banerjee et al. 2007; Cheng et al. 2012; Marques et al. 2014). Researchers have only just begun to identify epigenetic linkages between prenatal exposure to these toxicants and potential health risk among offspring. For example, one study among 247 infants in Mexico finds that maternal lead exposure during

pregnancy was associated with greater *HSD11B2* methylation in infants (Goodrich et al. 2015). These findings are underscored by another recent genome-wide association study where prenatal exposure to manganese was associated with DNA methylation to several genes related to neurodevelopment (Maccani et al. 2015).

As this section of the chapter summarizes, epigenetic alterations to the HPA axis can occur in utero and are sensitive to a range of exposures, particularly related to prenatal stress and maternal mental health. However, for such molecular changes to be truly indicative of future disease in the offspring, we must have evidence that gestational HPA-related epigenetic alterations are in turn associated with postnatal neurobehavioral and neuroendocrine risk markers. We now turn toward the evidence linking gestational *NR3C1* and *HSD11B2* epigenetic alterations to such postnatal phenotypes. We also summarize these findings in Table 5.2.

5.5 Gestational Methylation to *NR3C1* and *HSD11B2* and Offspring Neurobehavior and Neuroendocrine Regulation

Several studies have demonstrated that gestational *HSD11B2* and *NR3C1* methylation are associated with offspring neurodevelopment and neuroendocrine regulation. The vast majority of the studies in this area have focused on epigenetic alterations to *NR3C1*. Some of the most recent compelling evidence linking gestational epigenetic alterations of these HPA-axis genes to neurodevelopment and neuroendocrine function come from the Rhode Island Child Health Study (RICHS), a large population-based study of nearly 800 healthy pregnant women and their term infants from Providence, Rhode Island. RICHS investigators have focused on identifying epigenetic alterations in the placenta to help explain the developmental origins of mental disease risk as the fetal environment is regulated by the placenta, which plays an active immunoendocrine functional role in pregnancy, in addition to its role in nutrient, gas, and waste exchange (Paquette et al. 2013). The placenta is the first complex fetal organ to form and is involved in the development of the child's HPA axis; it thus plays an integral role in neurodevelopment and laying the foundation of neuroendocrine function.

In RICHS, neurodevelopment was measured within days of birth with the Neonatal Intensive Care Unit Network Neurobehavioral Scale (NNNS), a validated tool for the assessment of infant neurobehavior (Lester and Tronick 2004a, b). The predictive capability of this tool for identifying future mental and behavioral morbidity is robust as past work has shown neonatal neurobehavior measured by the NNNS to prospectively predict neurological problems, behavior problems, and cognitive function in later childhood (Liu et al. 2010; Stephens et al. 2010; Tronick and Lester 2013). The NNNS is administered by a trained psychometrician, takes 25 minutes to complete, and provides summary information in 13 neurobehavioral domains: attention, quality of movement, self-regulation, habituation, stress/abstinence, arousal,

Table 5.2 Studies examining association between gestational *NR3C1* and/or *HSD11B2* methylation with postnatal neurobehavioral and neuroendocrine outcomes

Study	Population	n	Gestational epigenetic alteration	Postnatal phenotypic outcome
Appleton et al. (2015)	Healthy term pregnancies, Providence, Rhode Island, USA (RICHS)	372	Placental *NR3C1* and *HSD11B2* methylation	Patterning across 3 NNNS domains of neurobehavior at birth (habituation, excitability, asymmetrical reflexes)
Bromer et al. (2013)	Healthy term pregnancies, Providence, Rhode Island, USA (RICHS)	186	Placental *NR3C1* methylation	Worse NNNS-assessed quality of movement and attention at birth
Conradt et al. (2013)	Healthy term pregnancies, Providence, Rhode Island, USA (RICHS)	521	Placental *NR3C1* and *HSD11B2* methylation	Worse NNNS-assessed self-regulation, hypotonia, and lethargy at birth
Conradt et al. (2015)	Healthy population-based sample from Rhode Island, USA	128	Placental *NR3C1* methylation	Dysregulated cortisol reactivity and self-regulation in response to laboratory-based stressor at 5 months of age
Marsit et al. (2012)	Healthy term pregnancies, Providence, Rhode Island, USA (RICHS)	185	Placental *HSD11B2* methylation	Lower birth weight and worse NNNS-assessed quality of movement at birth
Oberlander et al. (2008)	Women with depression and healthy controls from Vancouver, Canada	82	*NR3C1* cord blood methylation	Dysregulated salivary cortisol response to a laboratory-based stressor at 3 months of age
Stroud et al. (2014)	Healthy term pregnancies, sociodemographically diverse sample in Providence, Rhode Island, USA	45	*NR3C1* cord blood methylation	Dysregulated salivary cortisol response to a laboratory-based stressor over the first month of life
van Mil et al. (2014)	Dutch cohort with oversampling of children with ADHD (Generation R Study)	426	*NR3C1* cord blood methylation	No association with symptoms of attention deficit hyperactivity disorder at 6 years of age

handling, excitability, lethargy, hypertonicity, hypotonicity, nonoptimal reflexes, and asymmetric reflexes (Lester and Tronick 2004b). Next, we review notable findings from the RICHS cohort for gestational DNA methylation to *NR3C1* and *HSD11B2* in relation to infant neurobehavioral risk as assessed with the NNNS.

In two examinations of the first 186 RICHS infants, greater placental DNA methylation extents of *NR3C1* and *HSD11B2* were associated with worse newborn

neurobehavior in terms of quality of movement and attention (Marsit et al. 2012; Bromer et al. 2013). These findings were maintained when accounting for birth size for gestational age, infant sex, and maternal age; these studies also demonstrated less placental gene expression according to greater methylation extents to each gene. A more recent analysis among a larger RICHS subset ($n=521$) found greater *NR3C1* and *HSD11B2* placental methylation to be each associated with worse infant neurodevelopment in terms of self-regulation, hypotonia, and lethargy, particularly among newborns whose mothers had either depression or anxiety during pregnancy (Conradt et al. 2013). RICHS investigators have also examined gestational epigenetic alterations to other HPA-axis genes and effects on infant neurobehavior. These studies have found greater degrees of placental DNA methylation to the serotonin receptor (*HTR2A*; regulator of fetal brain development, linked to later life mood, aggression, anxiety) (Paquette et al. 2013), *FKBP5* (linked to posttraumatic stress disorder, depression, anxiety) (Paquette et al. 2014), and leptin (*LEP*; related to energy expenditure and metabolism) (Lesseur et al. 2014a) to be prospectively associated with worse infant neurobehavior across a variety of domains, including attention, quality of movement, arousal, stress/abstinence, hypotonicity, and lethargy.

While an observational study, RICHS has several methodological strengths that underscore the validity of the findings. In all of the RICHS examinations, the NNNS assessment was administered within the first few days of life (at least 24 h after birth but before hospital discharge), therefore effectively capturing the prenatal programming effects for neurobehavior and excluding the possibility of confounding effects by postnatal influences. Moreover, the inclusion of largely healthy mother and infant pairs provided a robust examination of prenatal influences, placental epigenetic alterations, and infant neurobehavioral functioning that was not distorted by the physical health status of the mother. Finally, the investigators utilized a matching strategy at enrollment according to birth size for gestational age which controlled for confounding by being born small or large for gestational age; this approach also provides statistical power to examine methylation extents within these extreme birth size groups which otherwise would be low prevalence in a healthy population-based cohort. With these methodological strengths in mind, evidence from the RICHS cohort provides strong evidence for the intergenerational transmission of mental health risk by modification of the epigenome in utero. However, given that about half of the studies published to date in this area come from the RICHS cohort, replication and extension of these findings in other populations are critically important.

The observational findings from RICHS are congruent with exciting work in other samples that focused on gestational HPA-related programming and infant neurobehavior and neuroendocrine function using laboratory-based stress protocols. For example, Oberlander's (2008) study of maternal prenatal depression, *NR3C1* cord blood methylation, and infant cortisol response ($n=82$) discussed previously found greater methylation extent of cord blood *NR3C1* (CpG 3) to predict dysregulated salivary cortisol response to a stress challenge at 3 months of age (a habituation information-processing task that involves exposure to visual stimuli). These findings held after accounting for neonate characteristics, prenatal exposure

to serotonin reuptake inhibitor antidepressants, prenatal maternal mood, and postnatal maternal stress levels (Oberlander et al. 2008). Similarly, in a study of 100 healthy mother and infant pairs, biologically verified maternal smoking during pregnancy was found to contribute to blunted cortisol reactivity in response to the NNNS assessment over the first month of life; an exploratory analysis among 45 of the participants suggested that *NR3C1* cord blood methylation mediated the association (Stroud et al. 2014). Likewise, in another examination among 128 mother and infant pairs, placental *NR3C1* methylation (CpG sites 5–13) was significantly associated with cortisol reactivity and behavioral self-regulation at 5 months of age in response to a laboratory-based stressor designed for infants (the still-face paradigm) (Conradt et al. 2015). Taken together, these studies indicate that gestational methylation of *NR3C1* may be related to neuroendocrine function and markers of neurodevelopment, thus potentially setting the stage later for mental health risk.

It is important to note that not all studies have found significant associations between gestational *NR3C1* methylation and neurodevelopmental risk in childhood. One study among 426 Dutch children found no association between *NR3C1* methylation in cord blood and symptoms of attention deficit hyperactivity disorder at 6 years of age, although associations with methylation to the serotonin transporter (*5-HTT*) and dopamine receptor (*DRD4*) were noted (van Mil et al. 2014). This study suggests that while HPA-related epigenetic profiles identifiable at birth may associate with neurodevelopmental risk in later childhood, gestational dysregulation of *NR3C1* may not be the sole factor underlying the associations.

To our knowledge, only one study has considered the joint contribution of epigenetic alterations to both *HSD11B2* and *NR3C1* for infant neurobehavior (Appleton et al. 2015). Among 372 RICHS newborns, we tested the interaction between placental *NR3C1* and *HSD11B2* methylation in association with NNNS-assessed neurobehavior at birth. We found interactions between methylation of these genes in relation to distinct and uncorrelated domains of neurobehavior: habituation, excitability, and asymmetrical reflexes. Moreover, different patterns of DNA methylation across the cortisol regulation pathway predicted different neurobehavioral phenotypes. Those with low *NR3C1* methylation but high *HSD11B2* methylation had lower excitability scores; those with high *NR3C1* methylation but low *HSD11B2* methylation had more asymmetrical reflexes; and those with high methylation across both *NR3C1* and *HSD11B2* had higher habituation scores. These results suggest that epigenetic alterations across the cortisol regulation pathway may contribute to different neurobehavioral phenotypes, likely though varying degrees of glucocorticoid exposure during gestation. For example, infant excitability reflects physiologic and motor reactivity to stimuli and control over body systems in the presence of stressors. We found that lower excitability scores were associated with low *NR3C1* and high *HSD11B2* methylation. In this sample, higher methylation of these genes is associated with lower expression (Bromer et al. 2012; Marsit et al. 2012). Thus, the pattern of DNA methylation we observed for excitability may suggest reduced expression of *HSD11B2* and greater *NR3C1* expression, which could in turn be associated with increased fetal exposure and response to cortisol.

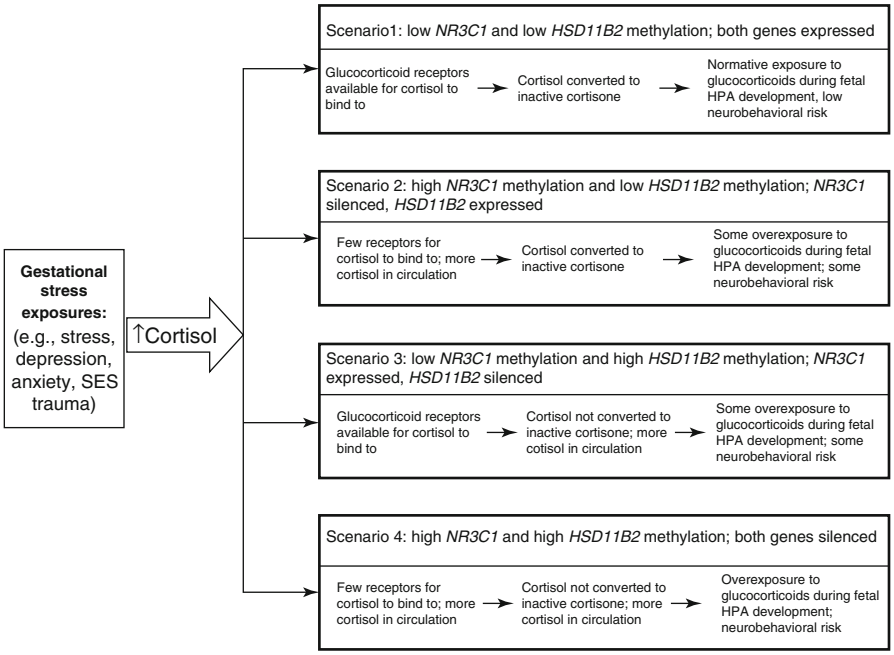

Fig. 5.2 Alternative scenarios for gestational epigenetic programming of the cortisol regulation pathway and neurobehavioral risk

This study underscores findings from the single candidate-gene analyses that largely characterize the field of gestational HPA-related epigenetic effects on infant neurobehavior and neuroendocrine function. However, each gene is part of a complex and integrated biological system and, as such, should be considered in conjunction with one another. As we observed in the previously reviewed study, it is possible that epigenetic patterning across genes involved in the regulation of cortisol could contribute to differences in neurobehavior observable at birth. Figure 5.2 depicts four broad alternative scenarios that help describe how gestational methylation and resultant gene expression patterns for *NR3C1* and *HSD11B2* could simultaneously and differentially affect neurobehavioral risk. For example, scenario1 depicts a low degree of methylation across *NR3C1* and *HSD11B2*, which indicates that these genes are fully expressed, and in turn the developing fetus is exposed to normative levels of cortisol during HPA development. We would expect neuroendocrine and neurobehavioral risk to be low in such a scenario. In contrast, scenario 4 depicts a high degree of neurobehavioral risk as both *NR3C1* and *HSD11B2* are highly methylated, indicating low expression of both genes, resulting in overexposure of glucocorticoids during fetal development due to dysregulation of the entire cortisol regulation pathway. Scenarios 2 and 3 depict moderate levels of risk as one but not both genes are highly methylated; some part of the cortisol regulation pathway remains functional. These scenarios are simplified heuristics to underscore our point that these genes may be jointly operating in a variety of ways to affect neurobehavior, neuroendocrine function, and mental health risk. We encourage

future work to consider the simultaneous influence of epigenetic alterations to multiple genes to better and understand the developmental origins of mental health risk.

5.6 Gaps in the Evidence Base and the Way Forward

The sensitivity of the epigenome holds tremendous promise for understanding how deleterious exposures affecting one generation can become biologically embedded to affect health in subsequent generations. In this chapter, we have reviewed the evidence linking prenatal exposures to epigenetic alterations to two HPA-axis genes involved in the regulation of cortisol and related evidence highlighting how methylation to those genes during gestation in turn affects neurobehavioral and neuroendocrine risk among offspring. This is a promising field of research and may one day help inform psychiatric clinical practice and the prevention of mental disease. However, the field is in its infancy and many gaps in the evidence base remain. While the evidence is accumulating, it is premature to move toward translating the findings in neuroendocrine epigenetics to health management or guiding therapeutic decisions for individuals with mental disorders. Instead, we propose a research agenda that broadens the evidence base, which in turn will provide the first steps toward incorporating epigenetic signatures into clinical practice. We now turn toward explicitly outlining some gaps in the field, and new research approaches to bridge those gaps.

As we have summarized previously, epigenetic alterations to *NR3C1* and *HSD11B2* can occur in utero and are sensitive to a range of exposures, particularly related to prenatal stress and maternal mental health. However, there has been no examination of the epigenetic effects of multiple exposures occurring simultaneously. Exposures like prenatal psychosocial stress, tobacco use, pregnancy complications, and environmental toxicant exposures tend to be correlated, particularly among vulnerable populations, and these factors likely jointly contribute to the epigenetic modulation of *NR3C1* and *HSD11B2* among offspring. It is unlikely that the etiology of complex major mental health conditions like depression, schizophrenia, and posttraumatic stress disorder can be traced to one single exposure or epigenetic alteration to a single gene. In addition to continuing to characterizing the gestational epigenetic effects to *NR3C1* and *HSD11B2* from novel exposures, it is advised that future research move beyond examinations of single risk factors and single genes and consider the joint contribution of multiple exposures to epigenetic alterations across the cortisol regulation pathway. To that end, our group has recently implemented the Albany Infant and Mother Study, which is a prospective examination of pregnant women and their infants that centers on evaluating the joint contribution of multiple prenatal psychosocial, environmental, nutritional, and behavioral exposures with gestational epigenetic alterations to several interrelated HPA-related genes and neonatal outcomes among a disadvantaged population. We will characterize the multiple synergistic and cumulative influences operating during gestation that together shape epigenetic pathways and neuroendocrine risk. This study is just one example of a more comprehensive way of thinking about the intergenerational transmission of mental health risk.

Another gap to address relates to the study population. Much of the evidence linking prenatal exposures to HPA-related epigenetic alterations comes from RICHS cohort, a largely non-Hispanic white US population, as well as from other small, specialized populations from around the world (e.g., Belgium, Congo). While providing compelling contributions to the field, researchers should also extend this work to include disadvantaged populations subject to health disparities, such as African-Americans, Native Americans, Hispanic/Latinos, and low socioeconomic status individuals. Very little work in this area has focused on these groups, despite their being at disproportionate risk of experiencing deleterious prenatal exposures, poor perinatal outcomes, and having a greater burden of mental disease over the life course. There is a significant need to identify the biological mechanisms that help explain the disproportionate burden of disease experienced by racial/ethnic minorities and the poor. We may find that the epigenetic mechanisms heretofore observed are also relevant among disadvantaged populations, or we may find that entirely new epigenetic signatures and exposures help to explain transmission of disease risk across generations for these groups. In order to prevent and ameliorate health disparities, we must pursue this work to better characterize how health mental disease risk is transmitted across generations among vulnerable groups.

Finally, the vast majority of the evidence supporting the intergenerational transmission of mental disease risk via gestational *NR3C1* and *HSD11B2* methylation focus on observed neuroendocrine and neurobehavioral effects in infancy. While we make the assumption that such programming effects identifiable early in life are indicative of increased mental health risk over the offspring's life course, we simply have very little prospective evidence supporting this claim. Moreover, the epigenome and the development of glucocorticoid pathways remain tremendously sensitive to modulation in the postnatal environment, particularly in the early years of childhood and in the context of stress and relational exposures (Weaver et al. 2004; Meaney and Szyf 2005). In other words, postnatal exposures may exacerbate and also remediate risky epigenetic signatures identifiable at birth. In this vein, one recent UK study ($n = 181$) found infant *NR3C1* methylation at 14 months to be elevated in the presence of increased maternal postnatal depression following low prenatal depression; this deleterious effect was reversed by maternal stroking and cuddling of the infants over the first weeks of life (Murgatroyd et al. 2015). As the field continues to move forward, we advise future research to incorporate longer follow-up intervals to assess the long-term neuroendocrine and mental health impacts of gestational epigenetic alterations to the HPA axis. We also suggest that future work examine whether and how those epigenetic signatures indicative of neuroendocrine and neurobehavioral risk identifiable at birth are modulated by the postnatal environment.

5.7 Conclusion

The convergence of evidence around *NR3C1* and *HSD11B2* suggests that gestational epigenetic modulation to these gene promoters may be critical in linking neurobehavioral and neuroendocrine risk from mother to child. While we

acknowledge that the programming of mental conditions extends well into childhood, the work reviewed in this chapter provides important insights into the molecular basis for the gestational origins of mental conditions. The great promise of epigenetics, particularly in regard to the intergenerational transmission of mental disease risk, is the possibility that we will be able to identify the molecular mechanisms responsible for mental illness alongside modifiable environmental and behavioral drivers of risk. In so doing, this work will ultimately enable us to better treat and prevent psychiatric conditions, both within and across generations.

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Chapter 6
Intergenerational Effects of PTSD on Offspring Glucocorticoid Receptor Methylation

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Abstract Posttraumatic stress disorder is precipitated by the experience of extreme stress in a subset of vulnerable individuals. Data now suggest that PTSD in parents confers vulnerability to PTSD in offspring. This is underlined by the observation that offspring born to individuals with PTSD exhibit low baseline cortisol levels, a neuroendocrine risk factor for PTSD. Several mechanisms have been proposed to mediate intergenerational inheritance of PTSD, including genetics and social learning. However, there is also the possibility that biological changes associated with PTSD in parents directly impact offspring, potentially via changes to gametes, fetuses in utero, or via changes in early postnatal care. The exact mechanism of this form of "intergenerational transmission" is unknown; however changes in epigenetic signatures, including DNA methylation, provide an appealing candidate. Animal studies have prompted investigation into methylation of the *NR3C1* promoter, which regulates transcription of the glucocorticoid receptor gene. Here, we review evidence of *NR3C1* methylation alterations in offspring of parents exposed to extreme stress and offspring of parents who developed PTSD in response to extreme stress. We propose several hypotheses based on the current evidence and suggest future directions related to these interpretations.

Keywords PTSD • CpG island • Glucocorticoid receptor • Stress • Offspring • Methylation

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6.1 Introduction

Posttraumatic stress disorder (PTSD) manifests after extreme stress in a vulnerable subset of exposed individuals and is characterized by hyperarousal, avoidance, and re-experiencing symptoms (American Psychiatric Association, and American Psychiatric Association. DSM-5 Task Force 2013). Psychiatric research has largely been devoted to characterizing factors that confer vulnerability to PTSD in the face of trauma, including risk alleles (e.g., *ADCYAP1R1*), early childhood adversity, and neuroendocrine factors, such as low cortisol (Yehuda et al. 1990; Ressler et al. 2011). Additionally, it has now been demonstrated that parental PTSD may act as a vulnerability factor in promoting PTSD in offspring, in that offspring are at greater risk for developing PTSD if one or both parents had PTSD (Yehuda et al. 2008). This finding is bolstered by evidence suggesting that healthy offspring of parents with PTSD exhibit lower levels of cortisol, a putative neuroendocrine risk factor for PTSD (Yehuda and Bierer 2008).

These findings have naturally prompted the question of how parental PTSD confers greater risk for PTSD in offspring. Genetics are an obvious candidate, but studies suggest that genetics are not categorically explanative in mediating the trajectory from parental PTSD to offspring PTSD, as maternal PTSD is a more potent mediator of PTSD in offspring compared to paternal PTSD (Yehuda et al. 2008). Additionally, PTSD could transmit to offspring by influencing parental behavior related to interactions with offspring. For example, adult offspring of Holocaust survivors report significantly higher levels of childhood trauma, particularly emotional abuse and neglect. Furthermore, offspring born to mothers with PTSD report lower maternal care, but higher maternal overprotection (McFarlane 1987; Yehuda and Bierer 2008; Field et al. 2011). Aside from the negative effects caused by exposure to early childhood adversity that might contribute to the development of PTSD, offspring might also model maladaptive PTSD-related behaviors, which could separately predispose offspring. There is also the possibility that biological changes associated with PTSD in parents could directly affect offspring. Cross-species studies suggest that changes to gametes, the gestational uterine environment, or changes to early postnatal care represent a viable form of transmission whereby PTSD might be inherited (Yehuda and Bierer 2008; Dias et al. 2015).

Epigenetics are the ideal candidate to mediate this form of transmission, as epigenetic processes are sensitive to environmental perturbation (e.g., trauma), yet generate long-lasting signatures (Weaver et al. 2004). Indeed, animal studies demonstrate epigenetic changes in gametes and brain across generations that associate with inherited behaviors (Dias and Ressler 2014). Particular focus has been dedicated to investigating epigenetic changes that influence glucocorticoid receptor expression, encoded by the *NR3C1* gene in humans, as it relates to intergenerational transmission of stress and PTSD for several reasons. Individuals with PTSD exhibit hypothalamic-pituitary-adrenal (HPA)-axis dysfunction, including increased glucocorticoid receptor sensitivity, where greater suppression of cortisol in response to dexamethasone is observed (Yehuda et al. 1990, 1993). HPA-axis dysfunction is now thought

to precede and, significantly, promote development of PTSD (Delahanty et al. 2000). In this way, parental PTSD may alter epigenetic marks surrounding *NR3C1*, contributing to HPA-axis dysfunction and thus development of PTSD in offspring. In fact, variation in early parental care has been shown to differentially methylate the glucocorticoid receptor promoter in rat pups, subsequently contributing to offspring long-term neuroendocrine and behavioral changes (Weaver et al. 2004). Taken together, these studies have prompted investigation into the contribution of *NRC31* methylation in mediating the transmission of PTSD from parent to offspring.

In this chapter, we review the evidence suggesting that *NR3C1* methylation is a mechanism by which ancestral experience is transmitted to subsequent generations; specifically we focus on transmission of PTSD and the effects of exposure to trauma. Although the data are limited, the existing evidence is exciting and begs further inquiry.

6.2 What Is Transgenerational Transmission?

The idea that psychopathology is heritable stems from the observation that specific psychiatric disorders tend to run in families. Indeed, multiple genetic studies have uncovered alleles that confer risk for psychopathology. Twin studies, in particular, underline the importance of genetics in mediating heritability of psychopathology. However, data from these studies also indicate that genetics explain a small percentage of variance regarding disease susceptibility, pointing to a large role played by environment.

Environment can directly impact individuals, most notably via the contribution of trauma to the development of posttraumatic stress disorder (PTSD). It is also likely that environment indirectly impacts individuals through direct effects on their parents. Again, this has been documented anecdotally and empirically with regard to PTSD (Spiegelman 1991). Offspring of parents affected by extreme stress and, subsequently, PTSD are at greater risk for PTSD, other forms of psychopathology, and other adverse mental and behavioral outcomes. In a foundational study, Solomon et al. observed that offspring of Holocaust survivors were more likely to develop PTSD after serving during wartime, despite being comparably healthy to their peers prior to military service (Solomon et al. 1988). This study, however, did not evaluate parental PTSD. Our laboratory has extended this line of research, reporting that parental PTSD, particularly maternal PTSD, in response to the Holocaust is associated with greater risk of PTSD in offspring (Yehuda et al. 1998, 2001).

One mechanism by which trauma could impact offspring, via effects on parental mental health, is through parenting behavior, e.g., corporal punishment, overprotection, etc. Moreover, children may model parent affect or behavior, replicating parental maladaptive responses, an idea that is conceptualized by "social learning theory" (Bandura 1977). Alternatively, psychopathology likely impacts parent biology, including changes to gametes and the gestational uterine environment in pregnant women. In this way, changes to parent biology may also influence biological changes in offspring, which could subsequently predispose offspring to the development of psychopathology. Parent biological changes resulting from stress

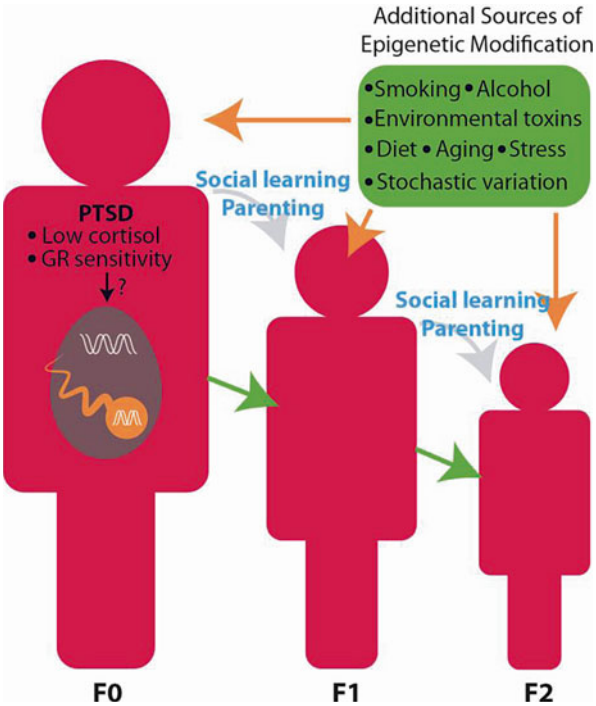

Fig. 6.1 "Intergenerational transmission" and other sources of epigenetic variation in offspring. One mechanism by which PTSD in parents could impact offspring is via maladaptive parenting behavior. Moreover, children can mimic disordered parent affect or behavior, an idea that is conceptualized by "social learning theory." Epigenetic effects in response to parenting and social learning are represented by *curved*, *gray arrows*. Additionally, environmental factors, including diet, stress, and aging, can modify the epigenome within each individual generation. Alternatively, psychopathology can impact parent biology (e.g., cortisol and glucocorticoid receptor sensitivity in PTSD). In this way, changes to parent biology may also influence biological changes in offspring, via epigenetic modification, which could subsequently predispose offspring to the development of psychopathology. Parent biological changes resulting from stress or psychopathology that impact their gametes, gestational uterine environment, or early postnatal care to ultimately alter offspring biology, and thus offspring outcomes, are referred to as "intergenerational transmission"

or psychopathology that impact their gametes, gestational uterine environment, or early postnatal care to alter offspring biology, and thus offspring outcomes, are referred to as "intergenerational transmission" (Fig. 6.1).

6.3 Epigenetics as a Mechanism by Which PTSD Is Transmitted

An obvious candidate mechanism underlying intergenerational transmission is epigenetic modification, an environmentally sensitive change that can alter gene expression (Zhang and Meaney 2010; Feil and Fraga 2011). Epigenetic modifications

include DNA methylation, histone acetylation, and histone methylation, among other marks. DNA methylation, as it relates to human psychopathology, has been the subject of particularly intense investigation. Focus on DNA methylation is likely due to methodological limitations related to storage of human specimens, as analysis of histone modifications would require cross-linking of fresh biological material. DNA methylation and demethylation involve the transfer or removal of methyl groups to cytosines in cytosine-phosphate-guanine (CpG) sites (Jones 2012; Klengel et al. 2014). Approximately 70 % of annotated gene promoters are associated with a CpG island, a 1000 base pair region with an elevated CpG composition (Jones 2012; Klengel et al. 2014). Heightened promoter methylation is typically associated with downregulation of gene expression and has been shown to interfere with transcription factor binding (Weaver et al. 2004; Klose and Bird 2006; Weber et al. 2007).

A critical impetus for the study of DNA methylation in mediating the transmission of stress from parent to offspring in humans comes from animal studies investigating early maternal care. These studies demonstrate that early maternal care programs adult offspring behavior where adult offspring of high licking and grooming and arched-back nursing (LG-ABN) mothers are less fearful and exhibit less exaggerated corticosterone response to stress than offspring of low LG-ABN mothers (Caldji et al. 1998; Weaver et al. 2004). Cross-fostering studies demonstrate that early maternal programming of offspring behavior is causal as biological offspring of low LG-ABN mothers reared by high LG-ABN dams exhibit behavior similar to biological offspring of high LG-ABN mothers. In a groundbreaking study, Weaver et al. report that maternal care programs offspring neuroendocrine and behavioral changes via methylation of the noncoding exon 1 promoter region of the hippocampal glucocorticoid receptor gene (*NR3C1*), where offspring of high LG-ABN mothers exhibit lower methylation compared to offspring of low LG-ABN mothers (Weaver et al. 2004). Lower methylation is associated with greater NGFI-A (also referred to as egr-1) transcription factor binding at *NR3C1*, greater GR expression, and a more modest HPA response to stress (Weaver et al. 2004).

Additional animal studies demonstrate that specific epigenetic marks are heritable across multiple generations and that these epigenetic marks are thought to mediate transmission of ancestral experience. For example, maternal separation is associated with depressive-like behavior in offspring (F1) and DNA methylation changes in F1 sperm (Franklin et al. 2010). Corresponding depressive-like behavior and DNA methylation changes are also observed in brains of the F2 generation (Franklin et al. 2010). Similarly, DNA hypomethylation surrounding *Olfr151*, a gene encoding the M71 odorant receptor, is observed in sperm of olfactory fear-conditioned F0 males and sperm of odor naïve F1 males (Dias and Ressler 2014). Importantly, F1 and F2 generations exhibit increased behavioral sensitivity to the F0-conditioned odor (Dias and Ressler 2014).

Altogether, aspects of epigenetics make it an appealing target mechanism to study in the mediation of intergenerational PTSD transmission. Demonstrated associations with transmission of ancestral experience in animals, along with methodological issues making human DNA methylation more amenable to study, have prompted the investigation of DNA methylation, specifically, in the transmission of PTSD in humans.

6.4 *NR3C1* Methylation Changes in Offspring of Individuals with PTSD

DNA methylation is responsive to the environment and changes to methylation as a reaction to some environmental factor can be stable over long periods of time, resulting in persistent changes to gene expression (Weaver et al. 2004; Zhang and Meaney 2010). As previously stated, these DNA methylation marks can remain stable, despite a shift in the environment originally responsible for initiating alteration of DNA methylation (Weaver et al. 2004). In this way, investigation of DNA methylation as a mechanism by which traumatic experience precipitates development of PTSD and then subsequently transmits psychopathology from parent to offspring is particularly attractive. Methylation changes to a small number of genes, including *SLC6A4*, *MORC1*, and *NR3C1*, are observed in offspring of individuals with PTSD (Nieratschker et al. 2014; Wankerl et al. 2014; Yehuda et al. 2014).

NR3C1, which encodes the glucocorticoid receptor, is a particularly promising candidate gene in the investigation of methylation changes in affected offspring. As mentioned, offspring of parents with PTSD are at greater risk for psychopathology and other behavioral and mental problems (Yehuda et al. 2001, 2008). Consistently, PTSD is associated with neuroendocrine alterations, including low baseline cortisol and greater glucocorticoid receptor sensitivity (Yehuda et al. 1990, 2004). Studies indicate that low cortisol is not solely an associated biomarker; rather, low cortisol appears to confer vulnerability for PTSD (Delahanty et al. 2000; Pineles et al. 2013; Walsh et al. 2013). As offspring of individuals with PTSD are more susceptible to the development of PTSD themselves, and, as expected, given that cortisol appears to be a risk factor for PTSD, they have lower levels of cortisol and greater glucocorticoid receptor sensitivity (Yehuda et al. 2000, 2002, 2007; Yehuda and Bierer 2008). These neuroendocrine alterations in offspring could be mediated by methylation changes to *NR3C1*.

The particular focus on *NR3C1* also stems from observed methylation changes at the hippocampal rodent ortholog in response to early maternal care (Weaver et al. 2004). Offspring of high licking and grooming and arched-back nursing mothers exhibit persistently lower hippocampal *Nr3c1* exon 1_7 promoter methylation, which is thought to program stable neuroendocrine and behavioral changes (Weaver et al. 2004). In humans, the *NR3C1* gene is comprised of 9 exons containing eight coding exons (exons 2–9) and multiple 5′-noncoding exon 1 splice variants. Together with exons D, J, E, B, F, G, C_{1-3}, and H, exon 1F and the exon 1F promoter form a CpG island containing two canonical and two noncanonical NGFI-A transcription factor binding sites (Turner and Muller 2005; Daskalakis and Yehuda 2014).

Given the results of the Weaver study, studies investigating intergenerational transmission of stress and psychopathology have focused on the *NR3C1* exon 1F promoter, the human ortholog of the rodent Nr3c1 exon 1_7 promoter. To date, there has only been one study examining offspring *NR3C1* methylation changes specifically in response to parental PTSD. In this cohort of Holocaust survivor offspring, an individual diagnosis of PTSD serves as an exclusion criterion in order to examine

an effect of parental PTSD on glucocorticoid receptor methylation, without the confound of offspring PTSD diagnosis (Yehuda et al. 2014).

Our laboratory observes a significant effect of parental PTSD on offspring *NR3C1* methylation; in particular, we find an interaction of maternal and paternal PTSD on promoter methylation averaged across 39 CpG sites. In these studies, the impetus for the investigation of parent gender and PTSD diagnosis on offspring *NR3C1* methylation stems from the previous finding that maternal PTSD is particularly potent in mediating offspring PTSD. Paternal PTSD is associated with higher levels of methylation, while offspring with both maternal and paternal PTSD exhibit the lowest levels of methylation (Yehuda et al. 2014). Differences in average *NR3C1* 1F promoter methylation levels according to parental gender and PTSD diagnosis appears to be mediated by individual methylation changes at specific CpG sites. Maternal PTSD is associated with hypomethylation at CpG 11, 23, and 30, whereas paternal PTSD is associated with hypermethylation at CpG 15 and 23. Differences in methylation at CpG 39 and 43 are associated with an interaction between maternal and paternal PTSD. Although maternal plus paternal PTSD is associated with hypermethylation of CpG 43, the presence of maternal PTSD appears to moderate an effect of paternal PTSD on hypermethylation, similar to previous findings (Daskalakis and Yehuda 2014). We did not observe an effect of Holocaust exposure on offspring *NR3C1* promoter methylation, suggesting that NR3C1 methylation changes are specific to PTSD (Yehuda et al. 2014).

Importantly, observed methylation changes are functionally significant. *NR3C1* 1F promoter methylation is negatively associated with *NR3C1* RNA expression, where, as expected, greater methylation is associated with less expression (Yehuda et al. 2014). Moreover, greater cortisol suppression in response to dexamethasone is associated with lower *NR3C1* 1F promoter methylation (Yehuda et al. 2014). In a separate study examining the same cohort, maternal PTSD associates with greater glucocorticoid sensitivity and paternal PTSD associates with decreased glucocorticoid sensitivity (Lehrner et al. 2014). These studies suggest that parental PTSD-mediated differences in glucocorticoid sensitivity according to parent gender could stem from changes in glucocorticoid receptor expression caused by differential promoter methylation. Although an exact mechanism by which parental PTSD is translated into differential epigenetic marks in offspring is unknown, animal studies suggest that DNA methylation changes in gametes in response to parental stress may precede later, more permanent methylation changes in offspring. It is important to note that offspring in the Yehuda studies were conceived years after the conclusion of the Holocaust, suggesting that differential methylation of *NR3C1* in offspring could be conveyed via gametes affected by biological changes associated with PTSD in parents.

Interestingly, data from studies investigating an association between *NR3C1* methylation and PTSD somewhat accord with offspring findings, where lower *NR3C1* 1F promoter methylation in peripheral blood mononuclear cells (PBMCs) is observed in combat veterans with PTSD compared with combat-exposed veterans without PTSD. Importantly, *NR3C1* 1F promoter methylation is similarly correlated with baseline cortisol levels and cortisol suppression, as in offspring, suggesting

that these epigenetic marks are functionally significant and related to HPA-axis dysregulation typically associated with PTSD (Yehuda et al. 2014). A separate study also finds *NR3C1* hypomethylation in individuals with PTSD, and this hypomethylation is associated with greater glucocorticoid receptor mRNA expression in whole blood (Labonte et al. 2014).

6.5 *NR3C1* Methylation Changes in Offspring of Stressed Parents

Compared to parental PTSD, relatively more is known regarding the effects of parental trauma and extreme stress on offspring NR3C1 methylation changes. These studies, however, focus on trauma that occurs during pregnancy, and thus they primarily examine a relationship between maternal stress and offspring *NR3C1* methylation. Three studies examining offspring *NR3C1* methylation in response to maternal experiences of intimate partner violence, genocide, and stress associated with war find increased methylation of the 1F promoter (Radtke et al. 2011; Mulligan et al. 2012; Perroud et al. 2014). These findings are in contrast to the PTSD data, where maternal PTSD is most often associated with offspring *NR3C1* hypomethylation (Yehuda et al. 2014).

Differences between the above-referenced trauma studies and the Yehuda PTSD study may account for conflicting methylation data. Most critically, differences in offspring methylation could reflect diverging epigenetic effects of trauma versus PTSD. Although mothers who experienced the Tutsi genocide were significantly more likely to develop PTSD, this study only differentiated between offspring of exposed and unexposed mothers (Perroud et al. 2014). A PTSD diagnosis was not assessed for the other two studies (Radtke et al. 2011; Mulligan et al. 2012).

Second, methylation changes may associate with period of transfer. For studies examining offspring *NR3C1* methylation in response to maternal experiences of intimate partner violence, genocide, and stress associated with war, trauma occurred during pregnancy (Radtke et al. 2011; Mulligan et al. 2012; Perroud et al. 2014). In contrast, Holocaust survivor offspring were conceived and born years after the end of the Holocaust (Yehuda et al. 2014).

Finally, although the Mulligan and Perroud studies observe an association between offspring *NR3C1* methylation and maternal prenatal stress, this does not exclude the possibility of a paternal stress effect on offspring *NR3C1* methylation. The Tutsi genocide and the Democratic Republic of Congo war were ongoing events that likely influenced fathers. Extreme stress effects in fathers could influence offspring *NR3C1* methylation in several ways. Paternal stress could cause biological changes in sperm which may be perpetuated in offspring. Sperm methylation and microRNA resulting from stress have been reported in animal studies (Franklin et al. 2010; Dias and Ressler 2014; Gapp et al. 2014). Additionally, stress in fathers could indirectly affect offspring by directly impacting pregnant mothers (fathers > pregnant mothers > offspring). Furthermore, prenatally stressed mothers

could impact fathers, further compounding stress effects in mothers (pregnant mothers > fathers > pregnant mothers > offspring).

There is also some evidence that less extreme forms of parental stress can impact offspring *NR3C1* methylation. Maternal pregnancy-related anxiety and prenatal cortisol are positively correlated with *NR3C1* methylation in offspring (Hompes et al. 2013). However, another study reports no differences in *NR3C1* methylation in prenatally stressed (defined as the sum of maternal psychological problems during pregnancy or the 3 months after delivery, preterm delivery, low birth weight, hospitalization of mother or child within 1 month after delivery, and maternal alcohol use or smoking during pregnancy) adolescent offspring (van der Knaap et al. 2014).

6.6 *NR3C1* Methylation and Other Forms of Psychopathology

There is also evidence that depressed mood in parents can impact offspring *NR3C1* methylation. Increased depressed maternal mood during the third trimester of pregnancy is associated with increased methylation at CpG 3 in *NR3C1* 1F of newborn cord blood. Furthermore, hypermethylation of this CpG site in newborns is associated with increased cortisol stress responses at 3 months old (Oberlander et al. 2008). Similarly, a separate study observes hypermethylation of placental CpG 2 of fetal origin related to maternal prenatal depression (Conradt et al. 2013). Interestingly, the authors find a significant interaction between maternal depression, placental CpG2 methylation, and neonate behavior, where infants of depressed mothers with greater CpG2 methylation exhibit poorer self-regulation and greater hypertonia (Conradt et al. 2013). This study is unique in relating maternal mood and offspring methylation status to offspring outcomes, suggesting that methylation effects may be functionally relevant.

6.7 Conclusion and Future Directions

Although the literature related to *NR3C1* methylation in offspring of individuals with PTSD is small, several conclusions can be drawn from the current data. Offspring *NRC31* promoter methylation appears to associate with parental stress exposure or parental psychopathology in response to stress, in that offspring of parents exposed to extreme stress exhibit *NR3C1* hypermethylation, while offspring of parents with PTSD exhibit hypermethylation or hypomethylation according to gender of the parent with PTSD and whether one or both parents have PTSD (Radtke et al. 2011; Mulligan et al. 2012; Perroud et al. 2014; Yehuda et al. 2014). Looking forward, it will be critical to determine whether *NR3C1* hypermethylation is protective in some way or dictates particular offspring outcomes apart from PTSD, which

may be driven preferentially by *NR3C1* hypomethylation. This is supported by data suggesting that paternal PTSD, which is associated with offspring *NR3C1* hypermethylation, is a less potent mediator of offspring PTSD compared to maternal PTSD (Yehuda et al. 2008).

Furthermore, it appears that parental PTSD preferentially mediates offspring PTSD, although this is somewhat contingent on parent gender as mentioned, while parental stress mediates other non-PTSD-related outcomes (Yehuda et al. 2008). Could this observation relate to offspring *NR3C1* methylation status, where changes in parent biology related to stress exposure versus psychopathology cause differential offspring *NR3C1* methylation, subsequently dictating diverging offspring outcomes? Data from studies investigating offspring born to prenatally stressed mothers find higher incidence of depressive symptoms and behavioral/emotional problems (O'Connor et al. 2002; Van den Bergh et al. 2008). Holocaust survivor offspring studies similarly observe increased vulnerability to anxiety, low self-esteem, inhibition of aggression, as well as depression (Yehuda et al. 2001; Gangi et al. 2009; Flory et al. 2011). Although offspring *NR3C1* methylation was not analyzed in these studies, it would be interesting to determine whether offspring of parents exposed to stress or trauma exhibit *NR3C1* hypermethylation, as we would expect given the data from the Perroud, Radtke, and Mulligan studies, among others, and whether this hypermethylation associates with non-PTSD-related offspring outcomes, including anxiety and depression.

Prospective studies may more definitively address this hypothesis to determine whether *NR3C1* methylation status directs offspring trajectories. Babies born to mothers who developed PTSD after exposure to the World Trade Center (WTC) attack on September 11, 2001, exhibit low baseline levels of cortisol (Yehuda et al. 2005). Although long-term offspring outcomes have not been measured, this finding lends support for the hypothesis that low cortisol and parent PTSD are risk factors for PTSD. In the same vein, *NR3C1* methylation in babies born to unexposed mothers, mothers exposed to the WTC attack, and mothers who developed PTSD in the aftermath of the WTC attack could be measured and then compared to subsequent offspring outcomes.

Assessing gamete *NR3C1* methylation in parents may more convincingly determine whether methylation is a mechanism rather than an associated biological change in offspring when trauma or psychopathology occurs prior to conception. Several rodent studies observe consistent epigenetic signatures in gametes and the brain across generations which parallel inherited behaviors, lending further support for epigenetics as a mechanism by which ancestral experience is transmitted (Franklin et al. 2010; Vassoler et al. 2013; Dias and Ressler 2014; Gapp et al. 2014). Studies of this nature may also clarify how these epigenetic changes are heritable, as these marks are thought to undergo reorganization during embryogenesis (Smallwood and Kelsey 2012). Similarly, fetal tissue-containing amniotic fluid collected via amniocentesis could be assayed for *NR3C1* methylation. Amniotic fluid has been used previously to predict offspring outcomes, where amniotic fluid cortisol was shown to associate with infant cortisol levels (O'Connor et al. 2013).

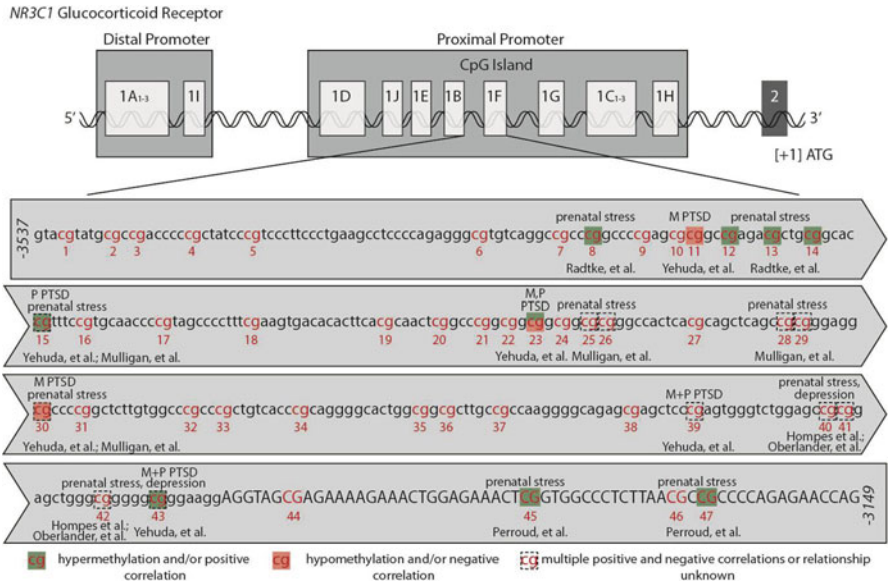

Fig. 6.2 Differentially methylated *NR3C1* 1F and 1F promoter CpG sites in response to parental stress, PTSD, and/or other forms of psychopathology. In humans, the *NR3C1* gene is comprised of 9 exons, with 14 exon 1 splice variants. Together with exons D, J, E, B, F, G, $C_{1–3}$, and H, exon 1F (*upper case letters*) and the exon 1F promoter (*lower case letters*) comprise a proximal promoter region located in a CpG island. CpG sites are numbered and highlighted in *red* (Daskalakis and Yehuda 2014). Reported offspring methylation changes to specific CpG sites in response to parental PTSD, stress, and/or depression are indicated by squares surrounding text (*green* hypermethylation or positive relationship between methylation and parental experience; *red* hypomethylation or negative relationship between methylation and parental experience; *dotted outline* multiple positive and negative relationships reported or relationship unknown). *M PTSD* maternal PTSD; *P PTSD* paternal PTSD; *M + P PTSD* interaction of maternal and paternal PTSD

Other additional methodological approaches may improve resolution of hypotheses regarding methylation changes around *NR3C1* in offspring. Studies are advised to measure average methylation and methylation at specific CpG sites along the *NR3C1* promoter. Furthermore, development of a consensus regarding a numbering system for specific CpG sites along the *NR3C1* 1F promoter and exon will be useful in determining consistency of methylation data across studies. For instance, CpG sites 15, 30, and 43 appear to be differentially methylated in offspring in response to parental PTSD, stress, and depression across three separate studies (Fig. 6.2 Consistent methylation changes across studies may indicate particularly sensitive or important CpG sites in mediating intergenerational transmission of ancestral experience.

Careful consideration of tissue type must also be taken into account. The studies reviewed in this chapter analyze *NR3C1* methylation in whole blood or PBMCs. DNA methylation signals in whole blood may be altered according to individual variation in cell type composition and degree of DNA methylation change (Lam et al. 2012). Fluorescence-activated cell sorting (FACS) may be useful in mitigating

DNA methylation variation stemming from different cell types. Nevertheless, tissue selection for methylation analysis will continue to require careful consideration, as new specimens and their relatedness to the brain are characterized (Smith et al. 2015). Regardless of methodological particulars, it will be important to continue to correlate *NRC31* methylation changes with glucocorticoid receptor expression to ascertain whether methylation differences are functionally significant.

Altogether, the data are provocative and suggest that methylation of *NR3C1* might be a mechanism by which risk for PTSD is transmitted from parent to offspring. Although more work is needed to replicate and extend the current findings, particularly across different populations, advances in the field make this an opportune time to address hypotheses related to the role of epigenetics as a mechanism by which ancestral experience is transmitted.

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Part III
Epigenetic Biomarkers and Neuroendocrinology

Chapter 7
Epigenetic Biomarkers for Early-Life Adversity

Gustavo Turecki

Abstract The early-life environment has important consequences on behavioral development. Specifically, the adversity experienced during sensitive developmental periods in childhood is associated with an increased risk for psychopathology in adulthood, such as mood disorders and mental illness. We have only recently started to uncover some of the mechanisms that may contribute to behavioral changes induced by early-life adversity. Epigenetics modifications regulate gene expression by changing the DNA's or chromatin's chemical composition or physical structure without altering its sequence. The epigenome is responsive to the environment, and landmark studies in rodents have shown that variations in early-life environment stably regulate the expression of key gene systems involved in behavioral and emotional responses through epigenetic processes. Early-life adversity could alter behavioral development and increase the vulnerability to psychopathology epigenetic regulation. This chapter will review the findings from human studies that support this hypothesis. We will describe epigenetic changes associated with early-life adversity and will discuss their potential as biomarkers for early-life adversity.

Keywords Child abuse • Epigenetics • Biomarkers • Psychopathology

7.1 Early-Life Adversity and Its Impact

Children in our society are all too often subjected to maltreatment, which is frequently perpetrated by their parents or caregivers. Accordingly, childhood maltreatment is a global problem of significant proportion that affects children of all ages, race, economic, and cultural backgrounds (US Department of Health and Human Services 2015). The four main types of childhood maltreatment include sexual

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abuse, physical abuse, psychological abuse, as well as parental neglect (Gilbert et al. 2009). From an epidemiological point of view, trauma exposure in children is estimated to range between 25 % and 45 %, although the rates reported vary considerably between studies and according to the definition of abuse types (Gorey and Leslie 1997; McCauley et al. 1997; Briere and Elliott 2003; Scher et al. 2004; Heim et al. 2010). The economic burden of child maltreatment and trauma resides mainly in its impact on the development of psychopathology later during adulthood. Indeed, child trauma, and particularly child sexual and physical abuse, is associated with increased risk of psychiatric disorders including depression, anxiety, bipolar disorder, substance abuse, and suicide (Fergusson et al. 1996; Santa Mina and Gallop 1998; Agid et al. 1999; Kaplan and Klinetob 2000; Kendler et al. 2000, 2004; Heim and Nemeroff 2001; Molnar et al. 2001; Evans et al. 2005). In addition to increasing the risk of psychiatric disorders, child sexual and physical abuse also associates with earlier age of onset of psychopathology, chronic course, more severe outcomes, and poorer recovery rates and, more importantly, with a 12 times higher odds of suicidal behaviors (Brown and Moran 1994; Bensley et al. 1999; Dinwiddie et al. 2000; Molnar et al. 2001; Zlotnick et al. 2001; Jaffee et al. 2002; Gladstone et al. 2004; Tanskanen et al. 2004).

While there is substantial evidence to support a link between childhood adversity and development of negative mental health outcomes in adulthood, a critical question that remains is the following: what long-lasting molecular changes occur as a result of the adverse life experience that could increase the risk for psychopathology? In this chapter we will review the data from human studies suggesting that changes at the molecular level are occurring in response to variation in the early-life environment. Specifically, we focus on epigenetic processes as they are thought to regulate expression levels of genes that affect response systems and, in turn, modulate behavior. Indeed, there is mounting evidence suggesting that epigenetic alterations in several key genes are occurring as a result of early-life adversity, and these changes may be contributing to increased risk for psychopathology. A discussion then follows on the potential use of these epigenetic markers as biomarkers for early-life adversity.

7.2 Epigenetics: Molecular Regulation by Environmental Factors

Although the genome contains the entire genetic information required to express all the proteins of an entire organism, only a fraction of this information is expressed in a given cell at a given time. Epigenetics refers to the collective chemical and physical processes that program the genome to express its genes in a time-and cell-dependent manner. They are dynamic molecular processes that regulate gene expression by changing the DNA's or chromatin's chemical composition or physical

structure without altering its sequence. These also include modifications that affect the availability of mRNA products. The epigenome is responsive to developmental, physiological, and environmental cues, and in this way epigenetics provides a basis for understanding how the environment may regulate the genome. Among epigenetic processes, the better characterized are DNA methylation, histone modifications, and noncoding RNAs.

The brain responds to life experience by adjusting its neuronal circuits and related functions, including behavior. Collectively, these brain responses to environmental stimuli are referred to as brain plasticity. While the brain always interacts with the environment, brain plasticity is not constant throughout the life cycle. There are periods in life, known as sensitive periods, during which the brain is more sensitive to the effect of experience (Nagy and Turecki 2012). Childhood is a sensitive period for behavioral regulation (Nagy and Turecki 2012), and there is growing evidence suggesting that epigenetic factors play a critical role regulating molecular mechanisms underlying brain plasticity during these sensitive periods (Baker-Andresen et al. 2013).

There is evidence to suggest that the early-life environment induces changes in stable epigenetic states that regulate gene expression and, ultimately, complex neural functions. The first evidence of this was demonstrated in studies investigating the effect of variations in maternal care in rats on stress reactivity. In both rodents and nonhuman primates, the early-life environment regulates hypothalamus-pituitary-adrenal (HPA) axis function in adulthood (Higley et al. 1991; Levine et al. 1994; Meaney 2001; Weaver et al. 2004). Landmark studies conducted by Meaney and colleagues demonstrated that variations in the early social environment, as modeled by maternal care in the rat (the frequency of pup licking/grooming (LG) over the first week of life), program the expression of genes that regulate behavioral and endocrine responses to stress. Specifically, these studies revealed that pups raised by mothers that exhibited increased frequency of pup licking/grooming (i.e., high LG mothers) exhibited in adulthood increased hippocampal glucocorticoid receptor (GR; *NR3C1*) expression, greater negative feedback regulation over hypothalamic corticotropin-releasing hormone (CRH), and more modest responses to stress compared to the offspring of low LG mothers (Liu et al. 1997; Francis et al. 1999; Weaver et al. 2004). Variations in maternal LG were found to be linked to an epigenetic modification of a neuron-specific exon 1_7 promoter of GR (Weaver et al. 2004) such that increased maternal care was associated with decreased methylation of the GR 1_7 promoter and increased hippocampal GR expression. This research underlines the profound and persistent impact that differential early-life experiences can have on gene expression and behavior through epigenetic mechanisms and DNA methylation changes, thus providing strong support for the ability of the early environment to stably influence neurodevelopment and complex behavioral traits. The exciting findings from animal studies have led to a growing interest in the investigation of epigenetic factors as possible mediators of biological embedding (Hertzman 2012) resulting from early-life adversity in humans.

7.3 Epigenetic Changes in Hypothalamus-Pituitary-Adrenal (HPA) Axis Genes Associated with Early-Life Adversity

Child abuse has been proposed to impose long-term effects on behavior partly by altering the neural circuits involved in the regulation of stress (Heim et al. 2008b). There is evidence to suggest that early-life adversity is associated with structural and functional alterations to several brain regions implicated in the stress response (Bremner et al. 1997, 2003; Stein et al. 1997; Driessen et al. 2000; Carrion et al. 2001; De Bellis et al. 2002). Individuals with a history of child abuse exhibited altered stress responses (Heim et al. 2000, 2008a), and individuals exposed to childhood abuse, particularly physical abuse, exhibit increased CRH levels (Carpenter et al. 2004; Heim et al. 2008b).

The findings in the rat showing that variation in early-life environment, measured by changes in levels of maternal care, induces wide epigenetic reprogramming in the brain, leading to altered regulation of stress reactivity into adulthood, were subsequently translated to humans. Accordingly, the first evidence for an effect of early-life adversity on the epigenetic state of the human genome was observed investigating the methylation state of the GR gene in the hippocampus of individuals who died by suicide and had histories of child abuse (McGowan et al. 2009). In particular early-life adversity in humans reprograms the DNA methylation patterns of one particular GR gene transcript variant, $GR1_F$ ($GR1_7$ homologue in rats) promoter. Increased methylation in the $GR1_F$ promoter region and decreased $GR1_F$ expression were found in the hippocampus of suicide completers with a history of child abuse compared to non-abused suicide completers and healthy controls (McGowan et al. 2009). This hypermethylation was associated with decreased binding of the transcription factor nerve growth factor-inducible protein A (NGFI-A), which likely accounted for the observed reduction in the hippocampal expression of GRs. Given that decreased GR expression is known to result in HPA axis hyperactivity, there is strong evidence implicating the role of childhood abuse in the disruption of this key stress-response system (Fig. 7.1).

This investigation was later extended to other transcripts of GR. The GR gene is preceded by noncoding exons, and in humans, nine first exon variants each possessing their own promoter region have been identified: $1_{A, I, D, J, E, B, F, C, \text{and } H}$ (Turner and Muller 2005) (Fig. 7.2). Expression of the noncoding exons 1_B, 1_C, and 1_H was found to be significantly decreased in hippocampus of suicide completers with a history of childhood abuse compared to non-abused suicides and controls (Labonte et al. 2012b). This assessment revealed that $GR1_C$ promoter methylation levels are inversely correlated with $GR1_C$ expression in accordance with the previous finding on the 1_F variant, whereas the $GR1_H$ promoter showed site-specific hypomethylation that was positively correlated with $GR1_H$ expression. This suggests that active demethylation is also a functional mechanism that could be affected by early-life adversity. While this is a mechanism that has received less attention, more work is required in order to elucidate its potential implications in the context of early-life adversity.

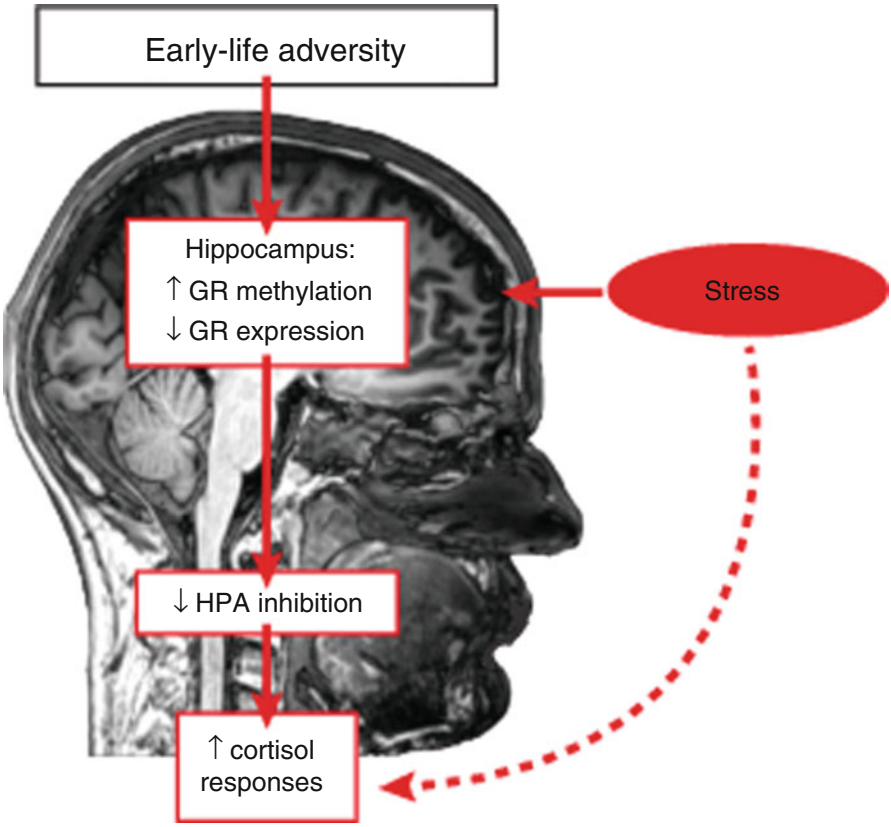

Fig. 7.1 Epigenetic programming of GR. Stress activates the HPA axis and causes the release of cortisol. The hippocampus provides negative feedback such that the binding of cortisol to hippocampal GRs causes inhibition of the HPA axis. Individuals that experienced early-life adversity exhibited increased GR promoter methylation and decreased GR expression in the hippocampus, which contributes to impaired HPA axis inhibition. During stress, poor cortisol GR binding hinders the negative feedback resulting in overactivity of the HPA axis

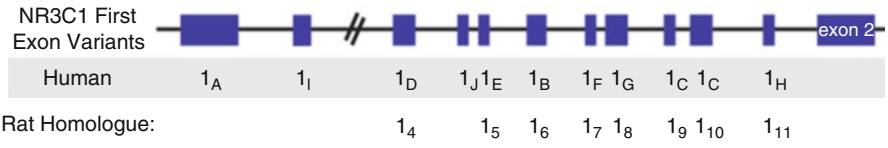

Fig. 7.2 First exon variants of the GR gene. In humans and rodents, the GR gene consists of 11 exons including untranslated first exon variants. Nine untranslated first exon variants each possessing their own promoter region have been identified in humans (A, I, D, J, E, B, F, C, and H) and in rats (1, 4–11)

These findings have been supported by several groups investigating peripheral blood samples from different populations of individuals that were exposed to varying forms of early-life adversity. Infants of mothers that reported suffering intimate partner violence during pregnancy exhibited higher levels of methylation in the promoter of $GR1_F$ compared to those born from mothers without such treatment (Radtke et al. 2011). In another study significant correlations were found between $GR1_F$ promoter methylation levels and parental loss, child maltreatment, and parental care such that diminished nurturing was associated with increased methylation of this gene (Tyrka et al. 2012). In addition, another group reported that childhood maltreatment, its severity of the type of abuse and frequency, was positively correlated with $GR1_F$ promoter methylation levels (Perroud et al. 2011). Altogether, the evidence seems to suggest that early-life adversity is capable of inducing specific long-lasting epigenetic alterations on the GR gene that ultimately affect its expression.

Another point of control of the HPA axis involves the activity of the FKBP5 chaperone protein, which (through the mechanism described below) decreases ligand binding to the GR, impeding its translocation to the nucleus (Scammell et al. 2001; Wochnik et al. 2005). FKBP5 is expressed when an activated GR binds to the glucocorticoid response elements (GRE) in FKBP5 gene enhancer regions. FKBP5 activity inhibits the GR-mediated negative feedback on the HPA axis, and the net result is a delay in the termination of the stress response. Several groups have reported that genetic polymorphisms in the FKBP5 gene interact with experiences of childhood abuse to predict adult post-traumatic stress disorder (PTSD), major depression, and suicide attempts (Binder et al. 2008; Roy et al. 2010; Appel et al. 2011). Klengel et al. (2013) studied peripheral blood samples from carriers of an FKBP5 risk allele (rs1360780) who were exposed to early childhood abuse and found these individuals exhibited decreased methylation near critical GRE regions of the FKBP5 gene. This single-nucleotide polymorphism was also shown to regulate the expression of FKBP5, suggesting that the HPA response system may be dysregulated in risk allele carriers. In short, the control of the HPA axis is complex and subject to several layers of regulation, which is critically adjusted by epigenetic mechanisms.

7.4 Epigenetic Changes in Brain-Derived Neurotrophic Factor (BDNF) Associated with Early-Life Adversity

Neurotrophins or neurotrophic factors have been gaining interest as candidate molecules to study in association with the development of psychopathology because of their role in neuronal survival and plasticity as well as the fact that they are expressed in the limbic areas of the brain, where emotions and related behaviors are processed. Of the major neurotrophic factors, brain-derived neurotrophic factor (BDNF), a neurotrophin involved in neuronal growth and development, has been the major focus in neurobiological research in relation to psychiatric conditions such as depressive disorders and suicide (Dwivedi et al. 2003; Brunoni et al. 2008; Pandey et al. 2008).

The role of early-life adversity in the epigenetic regulation of the BDNF gene was first investigated in a rat model of maternal care. Roth and colleagues studied the effect of repeatedly exposing pups for short periods of time to non-biological mothers exhibiting abusive maternal behaviors including pup avoidance and rough pup handling (Roth et al. 2009). Compared to controls, site-specific hypermethylation was found in the promoter region of transcripts IV and IX and decreased BDNF expression in the prefrontal cortex of the adult rats from the maltreated group. In addition, the deficits in BDNF expression were reversed by intracerebroventricular injection of a DNA methyltransferase inhibitor, supporting the involvement of epigenetic mechanisms in the regulation of BDNF expression.

The relationship between methylation of BDNF and early-life adversity has also been explored in human tissues. The human BDNF gene is composed of 11 exons preceded by 9 noncoding first exons regulating BDNF expression in different tissue (Pruunsild et al. 2007). The methylation state of the BDNF gene was characterized in human peripheral blood leukocytes from borderline personality patients who were assessed for childhood maltreatment (Perroud et al. 2013). In this population of patients, peripheral levels of methylation in the BDNF gene promoter increased as a function of the number of childhood traumas experienced, suggesting that methylation of BDNF associates with early-life adversity.

7.5 Epigenetic Changes in Serotonergic Genes Associated with Early-Life Adversity

Largely implicated in major depressive disorders and behavioral regulation, the serotonin system has also been studied in relation to epigenetic changes associated with early-life adversity. In particular, there have been several studies reporting on the serotonin transporter (5-HTT) gene, which has been implicated, among other things, in the interaction between early-life stress and the risk of depression (Caspi et al. 2003). An early study investigated in peripheral blood samples from rhesus macaques found increased methylation of the 5-HTT gene promoter associated with increased reactivity to stress in maternally deprived, but not mother-reared, infants (Capitanio et al. 2005).

Associations between early-life adversity and DNA methylation of the 5-HTT gene were also reported in humans. A significant association between childhood sexual abuse and overall DNA methylation of the 5-HTT gene promoter region was reported in an investigation of lymphoblast DNA samples from subjects of the Iowa Adoption Study (Beach et al. 2010, 2011). In addition, the DNA methylation patterns observed in this gene were associated with the emergence of antisocial personality disorder in adulthood (Beach et al. 2011). Another study using the same cohort of subjects also reported a correlation between childhood abuse and lymphoblast DNA methylation at four CpG sites in the nonpromoter regions of the 5-HTT gene (Vijayendran et al. 2012). The methylation status of the 5-HTT promoter was examined in peripheral blood samples of patients with major depressive disorders, and this was found to be significantly higher in those who experienced early-life adversity (Kang et al. 2013). On the other hand, a more recent study conducted in peripheral

blood from a German cohort failed to detect any site-specific changes in methylation associated with childhood trauma (Wankerl et al. 2014). The discrepancy in findings among different studies may have been due to the difference in the type of cells investigated and promoter subregions examined as well as the method used to measure methylation, which are important to take into consideration when making comparisons. Results from all the studies described above on DNA methylation changes associated with early-life adversity are summarized in Table 7.1 below.

Table 7.1 Summary of findings on DNA methylation changes and early-life adversity

Study	Gene	Experimental group	Tissue(s) analyzed	Results
Perroud et al. (2011)	GR	Childhood sexual abuse	Peripheral blood	↑ methylation of exon 1F promoter
McGowan et al. (2009)	GR	Suicide victims with history of child abuse	Hippocampus	↑ methylation at exon 1F promoter
Labonte et al. (2012b)	GR	Suicide victims with history of child abuse	Hippocampus Anterior cingulate gyrus	Studied promoter methylation of exons 1B, 1C, and 1H; changes found only in hippocampus: ↑ methylation at exon 1C, ↓ methylation at exon 1H
Radtke et al. (2011)	GR	Intimate partner violence during pregnancy (prenatal)	Peripheral blood	↑ methylation at exon 1F promoter
Klengel et al. (2013)	FKBP5	Childhood abuse (sexual or physical)	Peripheral blood	rs1360780 risk allele carriers show ↓ methylation near GRE in intron 7
Perroud et al. (2013)	BDNF	Childhood maltreatment (abuse and neglect)	Peripheral blood	↑ methylation of BDNF CpG exons IV and I as a function of the number of childhood traumas experienced
Beach et al. (2010, 2011)	5-HTT	Childhood abuse	Peripheral lymphoblast cell lines	↑ methylation in the promoter region (at early region of the promoter: CpG 1–17)
Vijayendran et al. (2012)	5-HTT	Childhood sexual abuse	Peripheral lymphoblast cells lines	↑ methylation at 4 CpG sites in nonpromoter regions
Kang et al. (2013)	5-HTT	Childhood adversities (parental loss, financial hardship, physical and sexual abuse)	Peripheral blood	↑ promoter methylation
Wankerl et al. (2014)	5-HTT	Childhood maltreatment	Peripheral blood	Studied CpGs in a promoter-associated CpG island; no changes were reported

7.6 Genome-Wide Epigenetic Changes Associated with Early-Life Adversity

The studies described above have all focused on the epigenetic changes occurring on a specific gene, but it is possible that early-life adversity may induce epigenetic adaptations across the entire genome. Labonte et al. (2012a) were the first to perform a genome-wide investigation of promoter DNA methylation in postmortem hippocampal tissue collected from individuals with a history of severe childhood abuse. Methylation profiles were compared between individuals that experienced severe child abuse and non-abused controls, and a total of 362 promoters were found to be differentially methylated in the abused group—248 were hypermethylated, while 114 were hypomethylated. Furthermore, the most significant DNA methylation changes were occurring mainly in neurons and specifically in genes that are implicated in neural plasticity.

More recently, several genome-wide studies investigating peripheral samples from individuals that experienced childhood maltreatment have been conducted. Studying peripheral blood samples obtained from post-traumatic stress disorder (PTSD) patients, Mehta et al. (2013) reported significant differences in the expression of several genes accompanied by greater instances of DNA methylation profile differences in the same genes in those who experienced childhood abuse than those without such histories. Another study that examined genome-wide promoter DNA methylation in peripheral blood from subjects of the 1958 British cohort found that childhood abuse was associated with significant differential methylation in 997 gene promoters with 311 that were hypermethylated and 686 that were hypomethylated (Suderman et al. 2014). The methylation differences were overrepresented in genes implicated in key cell signaling pathways related to transcriptional regulation and development. In addition, this investigation revealed significant DNA methylation differences in several genes that code for microRNAs in subjects with histories of child abuse compared to those without. A genome-wide investigation of DNA methylation in saliva samples obtained from abused or neglected children revealed that 2868 CpG sites were significantly differentially methylated in maltreated children compared to controls (Yang et al. 2013). The CpG sites implicated were located within genes purported to be involved in not only psychiatric disorders but also in several other health conditions commonly associated with childhood abuse including heart disease, stroke, and respiratory disorders among others. Altogether, the findings suggest that childhood abuse is associated with numerous epigenetic adaptations within the entire genome.

7.7 Epigenetic Marks as Biomarkers

It is evident that early-life adversity is associated with several epigenetic changes in genes that implicate a variety of biological systems. Although theoretically appealing, studying epigenetic changes caused by early-life adversity in humans and associating them with behavioral and emotional phenotypes pose a number of operational

and logistical challenges. Among these is the fact that epigenetic changes are cell and tissue specific, but sampling brain tissue from living subjects is not possible. This limits epigenetic studies to postmortem specimens, but the use of these tissues to study the neurobiological effects of early-life adversity has obvious limitations. While postmortem studies are certainly informative in many respects, it is difficult to establish temporal relationships between epigenetic marks and behavioral phenotypes from such studies. On the other hand, peripheral samples are much easier to obtain, but the extent to which they are informative on processes occurring in the CNS is a matter of current debate.

The use of epigenetic marks in peripheral tissues to study neuropsychiatric disorders has unclear scientific value as it is not clear to what extent epigenetic modifications in peripheral tissues are representative of those in the CNS. Epigenetic regulation is cell and tissue specific (Mikkelsen et al. 2007; Meissner et al. 2008; Lister et al. 2013), and there is greater epigenetic variability between different tissues from a single individual than between similar tissues from different individuals (Xin et al. 2010, 2011; Liang et al. 2011; Davies et al. 2012), which complicates the use of blood as a proxy to study brain disorders. To date, studies comparing peripheral epigenetic marks to those in the CNS have mainly focused on DNA methylation and have reported that DNA methylation patterns in a variety of brain regions are indeed reflected in peripheral blood (Dempster et al. 2011; Davies et al. 2012; Horvath et al. 2012; Masliah et al. 2013). These studies, however, are limited by their modest sample sizes and the fact that, in most cases, comparisons between blood and the brain were not made in samples collected from the same individuals. Therefore, the validity of using DNA methylation marks found in peripheral samples to study brain disorders remains unclear. There is still a need for larger-scale investigations in which brain and blood samples should be evaluated in the same individuals for direct comparisons to control for interindividual variation in genetic and environmental factors.

Although the relationship between central and peripheral DNA methylation marks remains unclear, the fact that there is significant consistency between studies investigating brain and peripheral DNA methylation associated with early-life adversity (as reviewed above) does suggest that systemic mechanisms may be at play. These processes, when occurring especially during a critical period of brain development such as during childhood, may lead to systemic changes in epigenetic patterns, which could then be also reflected in the periphery. For instance, early-life adversity activates stress responses that lead to the release of signaling molecules such as steroid hormones (e.g., glucocorticoids) or cytokines, which can act in multiple cell types and may lead to the initiation of a coordinated remodeling of the epigenome at specific sites. While this reasoning is still a hypothesis, it does suggest a pathway by which the epigenetic changes associated with early-life adversity might appear in a range of cell types. This would enable meaningful population analyses of the effects of childhood abuse on the epigenome. Furthermore, this suggests that the nature of specific epigenetic marks across multiple tissues might be dependent on the context and environmental conditions that may be capable of exerting sufficient biological impact (e.g., early-life adversity) and might lead to coordinated changes that would enhance the probability of detecting specific epigenetic states across multiple tissues.

In addition to DNA methylation, other mechanisms of epigenetic regulation have been uncovered and should be further explored in the coming years. For instance, recent studies investigating depression and the response to antidepressants have reported epigenetic changes in the brain that are also found in blood in histone marks (Chen et al. 2011; Lopez et al. 2013) and microRNAs (miRNAs) (Issler et al. 2014; Lopez et al. 2014), suggesting that these epigenetic marks may be useful biomarkers. The latter epigenetic mark, miRNAs, which play an important role in the posttranscriptional regulation of mRNA, is particularly interesting as there is evidence in humans that miRNAs also circulate in blood and other fluids and are stably transported in small double lipid-layered vesicles called exosomes (Valadi et al. 2007) or by lipoproteins such as HDL and LDL (Vickers et al. 2011). This exciting discovery suggests that circulating miRNAs in humans may act as molecular signals between cells and tissues and correlate with pathological states, and since then, there has been significant interest in the role of miRNAs as biomarkers of drug action and treatment response (Pappas et al. 2008) (Fig. 7.3). As growing data become available on epigenetic studies of early-life adversity using peripheral tissue samples, concerted efforts should be made to investigate the CNS validity of these findings. Although this is not an easy task, it will become easier once reference epigenomic maps are generated from different brain regions/cellular fractions in relation to maps from peripheral tissues.

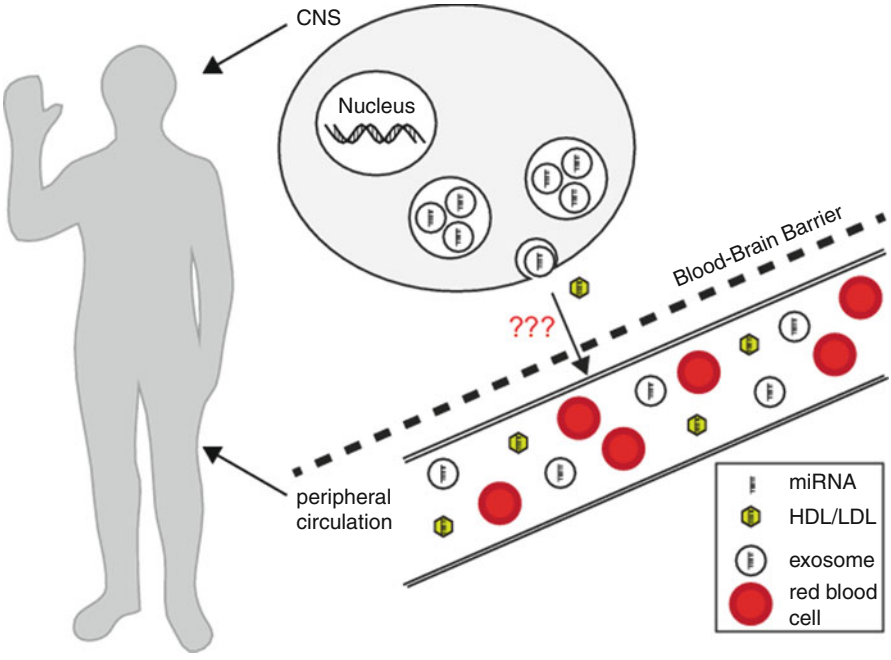

Fig. 7.3 Circulating miRNAs as biomarkers of CNS disorders. miRNAs are present in the peripheral bloodstream and are transported within exosomes or lipoproteins (HDL or LDL). It remains unclear whether miRNAs originating in the CNS can exit into the periphery. However, detecting changes in the levels of circulating miRNAs associated with a disorder would be a useful diagnostic tool

7.8 Conclusion

The studies reviewed here suggest that early-life adversity affects molecular mechanisms involved in the regulation of emotion and behavior. These effects involve changes in epigenetic regulation, which is believed to, by acting on genes involved in critical neuronal processes, be capable of inducing behavioral changes during development or later in life. The findings on postmortem brains and peripheral blood obtained from individuals with a history of childhood abuse described in this chapter point to several environmentally induced epigenetic alterations in the regulatory regions of genes involved in the response to stress, neuroplasticity, and neurotransmission associated with early-life adversity. These findings suggest that epigenetics may be a mechanism through which early environmental factors can induce long-term changes in behavioral responses, and these changes could have a negative impact leading to the increased risk to psychopathology. From a therapeutic standpoint, these epigenetic changes, several of which are detectable in peripheral blood, could be potential biomarkers used particularly for the development of clinical tools to identify at-risk individuals. The implementation, however, is not without its challenges as it is still necessary to clarify how representative peripheral epigenetic changes are of those occurring in the brain. The use of peripheral tissues such as blood could be a valid and promising method of examining the dynamic role of epigenetic adaptations implicated in mental disorders associated with early-life adversity, but further research here is still needed.

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Chapter 8
Epigenetic Biomarkers of Prenatal Maternal Stress

Fernanda Serpeloni, Karl M. Radtke*, Tobias Hecker, and Thomas Elbert

Abstract Diverse maternal experiences or mood disturbances before birth pose a substantial risk for poor lifetime mental health outcomes. DNA methylation variation in response to prenatal stress has been shown in animal model studies. Although prenatal time represents a sensitive period of development, little is known about the impact of maternal stress during pregnancy on DNA methylation during the life span in humans. In this review, we provide a brief summary of key human studies that bring evidence of DNA methylation in association with prenatal stress. We discuss common findings in the studies such as the type of maternal stress associated to offspring's DNA methylation and plasticity/stability of epigenetic variations. We also suggest the contribution of additional candidate gene approaches and genome-wide DNA methylation profile, in order to further explore and define the relationship between early social environment, epigenetics, and long-term outcomes. The implications of maternal care on DNA methylation as well as the importance of maternal well-being during pregnancy to prevent future health problems are considered.

Keywords Prenatal stress • Epigenetics • Maternal care • DNA methylation

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8.1 Prenatal Stress and Its Consequences for Health

Adversities affecting children during early development, even prenatally, pose major risk factors for psychopathological development, ranging from depression, anxiety, and substance use disorders to axis II diagnoses (personality disorders). Compared to non-maltreated individuals, psychiatric patients with a history of childhood maltreatment are characterized by earlier disease onset, greater symptom severity, more comorbidities, and poorer responses to treatments. Adversities during prenatal life have reliably proven to elicit long-term effects. Epigenetic modifications in stress-response systems, such as the hypothalamic–pituitary–adrenal (HPA) axis, may be a driving force producing these maltreatment-induced disorders. There appears to be limited specificity in terms of disease etiology, as various types of prenatal adversities have been associated with various types of outcomes. Factors include social exclusion, maternal mood disturbances such as those related to anxiety or depression, bereavement (Khashan et al. 2008), or the experience of natural- (Laplante et al. 2008) or man-made disasters (Yehuda 2005; Kleinhaus et al. 2013), daily hassles (Rice et al. 2010; Grizenko et al. 2012), smoking (Wakschlag et al. 2010; Cornelius et al. 2012), or malnutrition (Painter et al. 2006).

While most studies assess infant or child outcomes, manifestations of prenatal stress also occur in adulthood, highlighting the long-term impact of prenatal stress (Brown et al. 1995; Neugebauer et al. 1999; Painter et al. 2006; Entringer et al. 2008, 2009b; Buchmann et al. 2014; Betts et al. 2015). The diverse nature of stressor types is mirrored by a wide range of different health outcomes. Several studies report an association between prenatal stress and later behavioral or emotional problems, such as asocial behavior (Zohsel et al. 2014), hyperactivity disorder (Grizenko et al. 2012), aggression (Buchmann et al. 2014), or internalizing and externalizing behavior (Betts et al. 2015). Further consequences, the findings of which have been well replicated, include impaired cognitive function (Field et al. 2002; Buitelaar et al. 2003; Huizink et al. 2003; Niederhofer and Reiter 2004; King and Laplante 2005; Van Den Bergh et al. 2005; Mennes et al. 2006; Laplante et al. 2008; Entringer et al. 2009a) and severe psychopathological states such as schizophrenia (Susser and Lin 1992; Susser et al. 1996; Van Os and Selten 1998; Khashan et al. 2008) or autism (Beversdorf et al. 2005; Kinney et al. 2008). Finally, prenatal stress is associated with diverse findings such as sleep problems (Field et al. 2002; O'Connor et al. 2007), obesity (Ravelli et al. 1998, 1999), dermatoglyphic asymmetry (King et al. 2009), mixed handedness (Glover et al. 2004), or immune function (Wright et al. 2010; Veru et al. 2014).

Stress acts on several pathways such as the neuroendocrine and immune systems, which are in turn highly interwoven (Chrousos and Gold 1992). Stressful early-life events such as childhood physical and sexual abuse have been associated to long-lasting alteration of hypothalamic–pituitary–adrenal (HPA)-axis activity (e.g., Heim 2000; Carpenter et al. 2011; Schalinski et al. 2015). Furthermore, trauma-related disorders such as PTSD present with both a marked reduction of regulatory T cells (Sommershof et al. 2009) and altered brain function (Elbert et al. 2011).

Moreover, dysregulation of these pathways is associated with various diseases and behavioral problems (de Kloet et al. 2005). Accordingly, rather than focusing on a specific disease, many studies focused on investigating the physiological or neurological consequences of prenatal stress that place an individual at higher risk for disease development. Many studies report altered HPA-axis function in response to prenatal stress, which can be tilted the one way (elevated Diego et al. 2004; Huizink et al. 2008; Van den Bergh et al. 2008) or the other (flattening of the rhythm, Yehuda 2005; O'Donnell et al. 2013; Buchmann et al. 2014). Also brain development seems to be affected, as indicated by increased right frontal asymmetry (Diego et al. 2004), reduced gray matter density (Buss et al. 2010), or differential white matter microstructural organization (Sarkar et al. 2014). Finally, physiological conditions increasing the vulnerability for metabolic or cardiovascular diseases, such as insulin resistance, decreased glucose tolerance (Ravelli et al. 1998), or increased blood pressure (Painter et al. 2006), have been reported after prenatal stress exposure.

This wide range of health outcomes highlights the necessity of having a multidisciplinary, etiology-based approach in prenatal stress research, incorporating findings from fields such as mental health, immunology, and physiology in order to more accurately gauge the adverse effects of prenatal stress (O'Connor et al. 2014). Understanding how maternal experience in the social environment can be embedded in offspring's biology is of fundamental importance for creating effective interventions.

8.2 Adaptive Value of Prenatal Stress?

Via the placenta, which directly connects the in utero environment to the maternal physiology, the fetus receives signals about the maternal environment. Fetal programming denotes the concept that such signals can permanently alter the development of the fetus and the child. Although research has mainly focused on the adverse aspects in terms of increased disease susceptibility, this process is thought to be adaptive as it enables the fetus to acclimate to both its current environment and the one into which it will be born. Maternal constraint, i.e., mechanisms avoiding the outgrowth of the fetus from the pelvic channel, exemplifies a classic example of the adaptive values gained by maternal signals (Gluckman and Hanson 2004b). In the context of nutrition, the developmental (or fetal) origins of adult disease have been proposed. This theory states that poor nutrition during fetal development places the individual at higher risk for the development of cardiovascular diseases and metabolic disorders in adulthood (Barker 2004). Rather than exclusively attributing pathological consequences to physiological alterations induced by prenatal stress, the predictive adaptive response model assumes that these may be beneficial if the organism continues to be exposed to stressful conditions in later life (Gluckman and Hanson 2004a; Gluckman et al. 2005). However, if the in utero environment fails at predicting the future environment, the organism is at heightened risk for

developing unfavorable health conditions. Compelling evidence for this model was gathered when comparing the offspring of women pregnant during either one of two civilian famines of World War II, the Dutch Hunger Winter (1944–1945) and the Siege of Leningrad (1941–1944). In the former case, food was soon available again while the population remained starving in the latter case. Accordingly, in the Dutch cohort, those children were more susceptible to a range of metabolic diseases including insulin resistance or obesity while the latter cohort seemed to be unaffected (Stanner et al. 1997). These contradictory effects were hypothesized to result from fetal adaptations to a poorly nourished environment such as insulin resistance ensuring energy allocation toward the brain. While the aforementioned concepts and studies focus on maternal prenatal food availability and cardiovascular and metabolic consequences, the effects of prenatal stress on neurodevelopment are also likely to reflect a predictive role in assisting the individual to survive in a dangerous environment. From an evolutionary perspective, it has been debated, for example, that increased levels of aggression, a feature found to be associated with prenatal stress (Buchmann et al. 2014), may be adaptive in dangerous environments. However, in our modern society, we are usually not confronted with the types of stressors that demand an increased willingness for the display of aggressive behavior (for a review, see Glover 2011). Accordingly, adverse effects of incongruence between the pre- and postnatal environment have been found in a model of maternal depression. Infants of mothers experiencing inconsistent levels of depressive symptoms show signs of impaired motor and mental development, even though the incongruence was due to improving maternal mental health following delivery (Sandman et al. 2012).

8.3 Transmission of Prenatal Stress to the Fetus

A crucial question in understanding the contribution of the maternal well-being during gestation in determining the children's health concerns the transmission of maternal stress to the fetus, i.e., the biological correlate of maternal stress. The maternal HPA axis constitutes a likely candidate. Glucocorticoids are critical in normal development of the fetus as they are involved in the growth and maturation of many organ systems. However, exposure of the fetus to increased levels of cortisol, resulting, for example, from maternal stress, can lead to long-term "programming" of HPA function and behavior (Moisiadis and Matthews 2014). Indeed, elevated cortisol concentration in amniotic fluid was associated with blunted HPA-axis responses to acute stress and higher basal cortisol (O'Connor et al. 2013). Furthermore, it predicted decreased cognitive abilities (Bergman et al. 2010). However, due to the dynamics of placental CRH expression, the maternal HPA axis functions differently during gestation rendering

maternal cortisol exclusively mediating the effects of prenatal stress unlikely. In contrast to the cortisol-mediated feedback inhibition of the HPA axis, cortisol triggers placental CRH expression culminating in exponentially increasing maternal CRH and concomitantly cortisol levels over the course of pregnancy. Especially during later stages of pregnancy, the maternal HPA axis becomes less responsive, and the association between gestational stress and maternal cortisol levels is highly debated (for a review, see O'Donnell et al. 2009). Moreover, the placental enzyme 11β-dehydrogenase 2 (11β-HSD2) prevents the fetus from excess exposure to maternal cortisol by converting it into its inactive form corticosterone. Consequently, in utero cortisol levels can only partially reflect the mother's cortisol levels and are substantially lower (Gitau et al. 1998; Seckl 2004). Lower placental 11β-HSD2 expression causing increased in utero cortisol concentrations has also been suggested to be involved into the transmission of prenatal stress. Indeed, placental 11β-HSD2 expression has been shown to be downregulated by means of prenatal maternal stress (O'Donnell et al. 2012), which seems to be epigenetically regulated (Marsit et al. 2012; Conradt et al. 2013). A simplified model of the HPA and Placental Stress System during pregnancy is illustrated in Fig. 8.1.

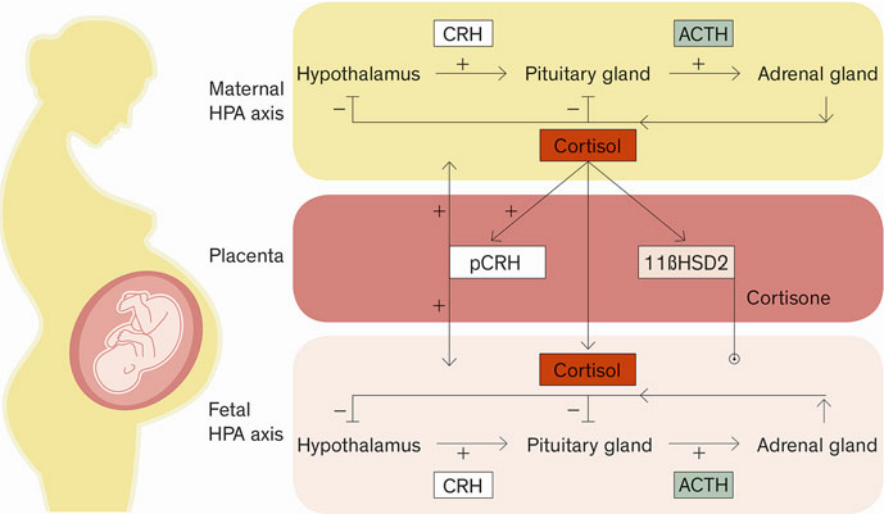

Fig. 8.1 During pregnancy, CRH is released from the placenta into both the maternal and fetal compartments. In contrast to the negative feedback regulation of hypothalamic CRH, cortisol increases the production of CRH from the placenta. Placental CRH (pCRH) concentrations rise exponentially over the course of gestation. In addition to its effects on pCRH, maternal cortisol passes through the placenta. However, the effects of maternal cortisol on the fetus are modulated by the presence of p11β-HSD2 which oxidizes it into an inactive form, cortisone (Modified version from Sandman et al. 2011)

The impact of maternal stress during pregnancy on the fetus involves complex pathways. In addition to the maternal HPA axis, other mechanisms are also considered to meditate the effects of gestational maternal stress. However, whether these can account for later health outcomes remains to be determined. For instance, reduced uterine blood flow potentially restricting fetal oxygen supply has been considered a possible correlate of prenatal stress (Teixeira et al. 1999). Via blood vessel constriction, maternal catecholamines released following distress may account for this phenomenon (Resnik et al. 1979). However, recent studies report only low or missing associations between prenatal maternal stress and uterine blood flow (Mendelson et al. 2011; Monk et al. 2012), thus questioning the latter as a correlate for maternal stress. Levels of inflammatory cytokines, which have been shown to be elevated in gestational stressed women (Coussons-Read et al. 2007; Blackmore et al. 2011), constitute another alternative. Indirect evidence for the implications of those in the offspring's development might arise from findings showing that maternal influenza in pregnancy is associated with schizophrenia (Brown et al. 2000).

8.4 Epigenetic Modifications: Molecular Mirror of the Prenatal Environment

Epigenetics involves a broad range of phenomena (dosage compensation, genomic imprinting) and mechanisms (chromatin organization and histone modifications) whereby DNA methylation is to date the most extensively investigated mechanism and the focus of this review. Although DNA methylation was thought to be restricted to early embryonic development, it has been shown to be plastic throughout life. Exposures to nutritional changes (Vucetic et al. 2010), chemicals (Onishchenko et al. 2008), and a broad range of environmental stressors (Weaver et al. 2004; Roth and Sweatt 2011) occurring during pre- and postnatal development have suggested that epigenetic regulation of gene expression is a critical target of experience-dependent changes. Epigenetic reprogramming, when occurring in primordial cells and during preimplantation development, leads to almost complete genome-wide DNA demethylation (Hackett et al. 2012). Nevertheless, evidence suggests that some epigenetic marks seem to persist across generations (Gapp et al. 2014). Furthermore, in line with the fetal programming concept, it has been debated that epigenetic modifications during the intrauterine period responding to adversities could serve as a possible genome adaptation mechanism, adapting the genome function to changes in the early environment, occurring in multiple tissues and across many regions in the genome. A model illustration of prenatal maternal stress, DNA methylation, and gene expression association is summarized in Fig. 8.2.

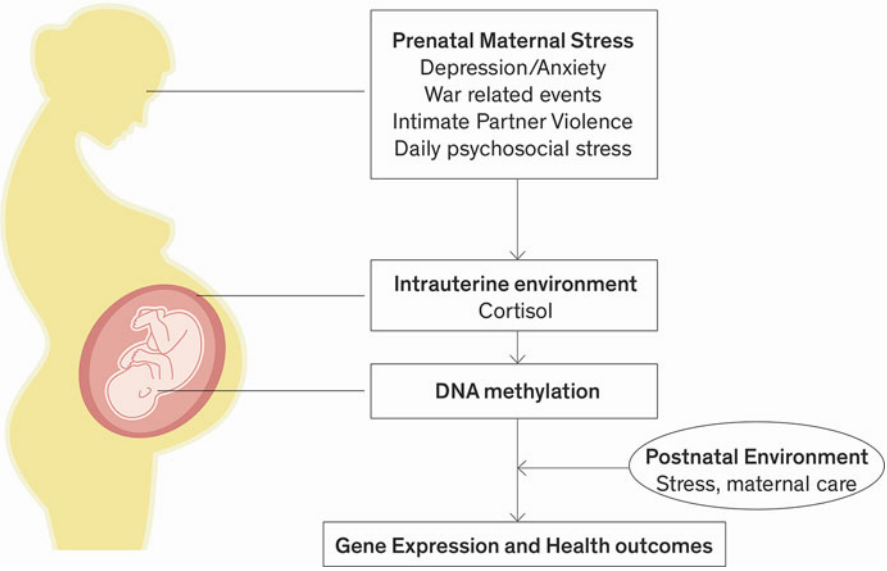

Fig. 8.2 Mood disorders (e.g., Oberlander et al. 2008), intimate partner violence (Radtke et al. 2011), war-related events, and daily psychosocial stress (Mulligan et al. 2012) have been reported in association with DNA methylation ultimately affecting gene expression and lifetime health. Furthermore, postnatal environment such as stress and maternal care are strong predictors of variations in DNA methylation (Weaver et al. 2004; Champagne 2008; Provençal et al. 2012; e.g., Tung et al. 2012)

8.5 Candidate Gene Approach

In candidate studies, the investigated genes are selected based on prior knowledge concerning their biological function or involvement in certain traits. The candidate approach allows for gaining further insights on the molecular and biological mechanisms that underlie the association between DNA methylation and the environmental stressors. For instance, it can provide information on whether a specific set of candidate genes is differentially methylated by means of early-life stress or on the effects of epigenetic modification on downstream processes such as gene expression.

A particular endocrine pathway affected by early adverse experiences is the "élite defense force" against stressors, i.e., the HPA axis. This pathway involves hormones secreted by the hypothalamus, pituitary, and adrenal glands as well as their receptors and plays a major role in stress responses. When functioning properly, the HPA axis helps in responding to a crisis. When unremitting stress forces the HPA axis to tilt one way, the result can be anything from a long-lasting head cold to depression. When tilted the other way toward a flattening of the rhythm of stress

hormones, the undesirable consequences may be abdominal fat, loss of muscle mass, and mental ailing (McEwen and Lasley 2002).

The first line of evidence in an association between early-life experience and changes in DNA methylation came from a candidate gene study focusing on the glucocorticoid receptor (GR) in rats. The GR is critically involved in the negative feedback regulation of the HPA axis. Weaver et al. (2004) showed that variation in maternal care during the first week of the rats' life regulates the level of methylation of the GR gene. In mice, the GR gene was also shown to be differentially methylated in response to prenatal stress (Champagne and Curley 2011).

The impact of prenatal stress on the methylation of the glucocorticoid receptor gene has also been investigated in humans. DNA methylation has been associated with different types of prenatal adversities, such as maternal mood disorders and traumatic events (Turecki and Meaney 2014), but also the absence of an effect has been reported (van der Knaap et al. 2014). In a prospective study, maternal suffering from depression symptoms was reported to be associated with both DNA methylation of the *GR* gene in neonatal cord blood and infant cortisol stress responses at 3 months (Oberlander et al. 2008). Conradt et al. (2013) extended this work using placenta tissue and found maternal depression during pregnancy associated with increased methylation of both the *GR* and the *11β-HSD2* gene suggestive of elevated intrauterine cortisol levels and thus a potential mechanism by which maternal stress is transmitted to the fetus. Accordingly, Hompes et al. (2013) reported an additive effect of gestational maternal cortisol levels and emotional state on *GR* methylation in cord blood. Recently, Murgatroyd et al. (2015) reported an interactive effect of prenatal and postnatal maternal depression. Methylation of the *GR* gene in infants was elevated in the presence of increased maternal postnatal depression following low prenatal depression. More interesting, those effects were reported to be reversed by maternal stroking of the infants over the first weeks of life (Murgatroyd et al. 2015). Furthermore, an association between war-related events during pregnancy and *GR* methylation in newborns has been shown (Mulligan et al. 2012). While the studies cited above investigated differential methylation at birth, these changes seem to persist into adulthood. In a retrospective study, we found that maternal exposure to intimate partner violence during pregnancy is associated with increased methylation of the *GR* gene in children aged 10–19 years old in peripheral blood samples (Radtke et al. 2011).

The methylation status of several other candidate genes have been investigated in association to adversities experienced during the prenatal period. For example in infants' cord blood, maternal depression during pregnancy was correlated with DNA methylation at the serotonin transporter gene *SLC6A4* but not *BDNF* (Devlin et al. 2010) as well as at the differentially methylated region (DMR) controlling the imprinted *IGF2*- and *H19*-genes (Chen et al. 2014). Another study investigating imprinted genes reported increased methylation at the *MEG3* DMR by means of gestational maternal depression or anxiety but not at the *IGF2* DMR (Liu et al. 2012). However, methylation of the *IGF2* DMR in this study was associated with low birth weight indicative of prenatal stress. Prenatal exposure to the Dutch Hunger Winter was associated with adult *IGF2* DMR methylation (Heijmans et al. 2008) and in the following genes *GNASAS*, *MEG3*, *INSIGF*, *IL10, ABCA1*, and *LEP* (Tobi et al. 2009). Perceived stress during pregnancy (Vidal et al. 2014) was associated

with higher infant DNA methylation at *MEST*, a gene relevant to abnormal maternal behavior and obesity in studies with mice.

While the majority of human epigenetic research investigating the effects of prenatal stress on DNA methylation focused on molecular findings, a few also incorporated phenotypic measures further implicating DNA methylation in mediating the association with prenatal stress. Differential DNA methylation evoked by prenatal stress was associated with low birth weight (Liu et al. 2012; Mulligan et al. 2012; Murphy et al. 2012), altered HPA-axis function (Oberlander et al. 2008; Yehuda et al. 2014), decreased GR expression (Yehuda et al. 2014), and impaired neonatal behavior and lethargy (Conradt et al. 2013). It has been suggested that the impact of environmental factors on the type and extent of epigenetic variations is dependent on the developmental stage of occurrence (Provencal and Binder 2015), and particularly early pregnancy (Heijmans et al. 2008; Tobi et al. 2009, 2014; Hompes et al. 2013) seems to play a critical role, although the third trimester has also been implicated (Oberlander et al. 2008). Interestingly, different types of adversities have been reported in association with offspring's DNA methylation: not only maternal exposure to severe traumatic events (e.g., war-related events) or mood disorders (e.g., depression/anxiety symptoms) but also more common prenatal stressful events, such as daily psychosocial stress (e.g., Mulligan et al. 2012; Vidal et al. 2014).

8.6 Epigenome-Wide Association Studies

With recent technological advancements, it is now possible to identify epigenetic variants across the genome and test their association with complex traits (Tsai et al. 2012). Inspired by the forward genetic approach of genome-wide association studies (GWAS), epigenome-wide association studies (EWAS) query DNA methylation at thousands of genomic locations in order to identify associations with a trait of interest. Unlike candidate gene approaches, a genome-scale analysis of differential DNA methylation enables the unbiased detection of new regulatory mechanisms that are susceptible to environmental changes. Many of these associated regions are unsuspected a priori. For example, DNA methylation in novel genes involved in diverse developmental processes that had not been previously implicated in responses to tobacco smoke was reported in association with tobacco smoke during pregnancy (Joubert et al. 2012). Non et al. (2014) performed an EWAS in umbilical cord blood of neonates exposed to maternal depression and anxiety during pregnancy and described a gene (*COL7A1*) linked with a skin condition (dystrophic epidermolysis bullosa) that had not yet been associated with depression or other psychological experiences. Interestingly biomarkers for prenatal adversity in genome-wide DNA methylation profiles have been reported also in adolescence and adulthood (Cao-Lei et al. 2014; Tobi et al. 2014). For instance, Cao-Lei et al. (2014) reported the patterns of DNA methylation in children whose mothers were pregnant when exposed to the Quebec ice storm in 1998 and found an association to pathways involved in the immune system 13 years later. DNA methylation changes in *SCG5* and *LTA*, both highly correlated with maternal objective stress, were

comparable in T cells, peripheral blood mononuclear cells (PBMCs), and saliva cells. Tobi et al. (2014) performed a genome-scale analysis in whole blood of individuals who were exposed to famine during early gestation at the Dutch Hunger Winter and identified key pathways related to growth and metabolism associated with malnutrition more than six decades later. Besides genes such as *GR* and *FKBP5* that are usually reported in the literature, we recently detected the association of DNA methylation with exposure to stress during maternal pregnancy in several other genes (e.g. *CNFN, STK32C, LHX9*), as well as grandmaternal pregnancy (e.g. *CORIN*) (Serpeloni et al., manuscript in preparation). Interestingly, as reported by the candidate gene studies, differentially DNA methylation was observed in different tissues (e.g., saliva, blood, T cells). The results support the "system wide" response hypotheses, based on evidence that phenotypic response to early-life adversity involves multiple phenotypes and thereby involves multiple systems (Szyf 2012). Concordantly, a recent study compared differential methylation by means of maternal lifetime depression in two cohorts using cord blood T lymphocytes and adult hippocampi. This study reported an overlap of 33 genes with changes in DNA methylation in the two tissues supporting the idea of lifelong epigenetic effects in various tissues (Nemoda et al. 2015). Table 8.1 summarizes the list of candidate gene and EWAS studies with humans reported in this review.

8.7 Maternal Rearing as Predictor of Variation in DNA Methylation

Timescale for maternal effects on epigenetic variations is suggested to not be restricted to the *intrauterine* environment. Although the prenatal period represents a sensitive period of development (Pirini et al. 2015), association of parenting and variations in DNA methylation is suggested to be extended to the entire life span, including before conception, such as nutritional and smoking influence on the gametes (for a review, see Lane et al. 2014) and postnatal period (e.g., Champagne 2008). Indeed parental care is a strong predictor of postnatal variation in DNA methylation (for a review, see Champagne 2008). It is known that maternal behavior can influence critical aspects of development such as offspring's neurobiology and behavior (Harlow and Suomi 1974; Sato et al. 1998; Taborelli et al. 2013). Evidence from experiments with animal models manipulating maternal care received by offspring has been reinforcing the plasticity idea of epigenetic states in response to variation in the social environment (Weaver et al. 2004; Provençal et al. 2012; Tung et al. 2012). For instance, as mentioned before in the experiment of Weaver et al. (2004), maternal care caused differential DNA methylation of the *GR* promoter region and correspondingly altered hippocampal GR expression and behavior. A cross-fostering design excluding genetic artifacts confirms that these effects were mediated by the quality of the postnatal environmental, i.e., maternal care. This provides a dynamic mechanism for maintaining long-term changes in the gene expression and behavior of offspring (Champagne 2008). Consistent with Weaver et al. (2004), rearing was associated with differential DNA methylation in an

Table 8.1 Prenatal stress and DNA methylation studies with humans

Gene	Study design	Maternal stress during pregnancy	Age of the offspring when DNA was collected	Tissue	Authors
GR	Candidate gene	Depression	Newborn	Cord blood	Oberlander et al. (2008)
IGF2	Candidate gene	Wartime famine	Six decades after famine prenatally exposure	Whole blood	Heijmans et al. (2008)
GNASAS, *MEG3*, *INSIGF*, *IL10*, *ABCA1*, *LEP*	Candidate gene	Wartime famine	51–62 years old	Whole blood	Tobi et al. (2009)
SLC6A4	Candidate gene	Depression	Newborn	Cord blood (leukocytes)	Devlin et al. (2010)
GR	Candidate gene	Intimate partner violence	10–19 years old	Whole blood	Radtke et al. (2011)
GR	Candidate gene	War-related events	Newborn	Cord blood	Mulligan et al. (2012)
MEG3, IGF2	Candidate gene	Depression	Newborn	Cord blood	Liu et al. (2012)
GR and *11β-HSD2*	Candidate gene	Depression, anxiety	Newborn	Placenta	Conradt et al. (2013)
GR	Candidate gene	Emotional well-being, cortisol	Newborn	Cord blood	Hompes et al. (2013)
SLC6A4, HSD11B2, CRHR2, EGR1, IGF2/H19, LUMA	Candidate gene	Depression, anxiety, perceived stress	Newborn	Placenta, umbilical cord blood	Chen et al. (2014)
MEST	Candidate gene	Perceived stress	Newborn	Peripheral, cord blood	Vidal et al. (2014)
Genes related to regulation of transcription, translation, and cell division processes	EWAS	Depression, anxiety	Newborn	Cord blood	Non et al. (2014)
Biological pathways prominently featured in immune function	EWAS	Ice storm	13 years old	T cells	Cao-Lei et al. (2014)
Biological pathways related to growth and metabolism associated	EWAS	Wartime famine	51–62 years old	Whole blood	Tobi et al. (2014)
Genes involved in immune system functions	EWAS	Depression	Newborn	Cord blood (T lymphocytes)	Nemoda et al. (2015)
GR	Candidate gene	Depression and postnatal maternal depression and stroking	5 weeks–14 months	Saliva	Murgatroyd et al. (2015)

experimental investigation of early maternal deprivation on adult rhesus monkeys (Provençal et al. 2012). Interestingly the differences in DNA methylation were observed in adulthood, supporting the hypothesis that the response to early-life adversity can persist into adulthood. Epigenetic changes during adulthood were also reported in female monkeys when exposed to experimentally controlled changes in social status (i.e., dominance rank) during adulthood (Tung et al. 2012). Moreover, when the monkeys moved to a new rank, their gene expression profiles changed as well, suggesting epigenetic flexibility in responses to the changes in the social environment (Tung et al. 2012).

In humans, the disruption or lack of adequate nurturing and inadequate parental care was associated with increased *GR* promoter methylation (Tyrka et al. 2012). In addition, epigenetic changes in the proopiomelanocortin (POMC) gene may promote HPA-axis dysfunction (Ehrlich et al. 2010). In a very recent study, we found that inadequate parental care and maltreatment was associated with children's differential methylation in the *POMC* gene measured in saliva and blood (Hecker et al. 2016). In a sample of former indentured child laborers who suffered severe childhood adversities functional annotation clustering suggested several gene clusters were differentially methylated (Marinova et al. 2015). The genes with hypermethylated CpGs were related to cellular morphogenesis, neuronal and cell development, and differentiation. Genes with hypomethylated CpGs were enriched in clusters related to impaired connectivity of neurons. The study indicated that distinct differences in DNA methylation associated with childhood trauma could be detected in late adulthood. These findings emphasize the detrimental consequences of inadequate parental care and child maltreatment.

8.8 Implications of Epigenetic Plasticity

The notion of epigenetic plasticity and thus the reversibility of epigenetically determined health conditions might open new avenues for therapy by either pharmacological agents or social interventions and the evaluation thereof (Szyf 2011). Specific drugs such as histone deacetylase inhibitor trichostatin A and methionine have been shown to reverse early experience-associated changes in DNA methylation and behavioral changes in adulthood (Weaver et al. 2006). Furthermore, a recent study suggested that the epigenetic state might be changed after psychotherapy (Yehuda et al. 2013). Therefore, at least certain trauma-associated epigenetic changes seem to be reversible, and positive environment might induce epigenetic variation that would impact the response to stress (Provencal and Binder 2015).

These observations point to a seemingly contradictory scenario. On the one hand, prenatal stress can have long-lasting impact on DNA methylation. On the other hand, epigenetic modifications are also sensitive to environmental changes throughout the life span. Whether the nature of epigenetic changes is dependent on windows of sensitivity or is specific to a genomic region (see Daskalakis and Yehuda 2014) and the complex interactions involved in methylation stability remains to be investigated. Although evidence of prenatal studies has shown an association

between fetal exposure and maternal stress, there is also a possibility that this influence is indirect. Maternal stress experienced during the prenatal period compromises maternal care during the postnatal period and thus influences offspring development (Champagne 2008).

As reviewed above, a variety of adverse conditions increase the risk for an impaired development of the children, as reflected at the molecular level. This implies a great burden not only on the affected individual but also on the greater society, e.g., due to the costs arising from the complications stemming from mental ill health and the associated mental health-care treatments (Soni 2009). Therefore, preventative interventions improving the well-being of pregnant mothers as means to circumvent future health problems in their children are crucial and most likely more economically beneficial than interventions targeting these children after unfavorable health conditions or molecular patterns have already manifested. While physical attributes such as diet or avoidance of toxin exposure have been incorporated and proven to be beneficial in the care of pregnant women (Kirkham et al. 2005), psychosocial risk factors have been largely neglected. Instruments to identify women confronted with psychosocial risk factors are fundamental in filling this void (e.g., Reid et al. 1998; Harrison and Sidebottom 2008; Austin et al. 2013; Spyridou et al. 2014; Spyridou and Schauer 2015).

Taken together, both candidate gene approach and EWAS can bring valuable contributions to the field. Focus on target genes contributes to the understanding of biological events that mediate connections between early stress and biological function. Furthermore, understanding methylation patterns at the level of specific candidate genes may be beneficial when assessing the effectiveness of clinical treatments. Describing the DNA methylation signatures from a genome-scale analysis associated with a trait is an important step in establishing DNA signatures of prenatal stress, uncovering new regulatory mechanisms, and providing a better understanding of complex pathways involved in different biological functions that are susceptible to environmental changes. Hence, knowledge of the factors that are involved in the stability and reversibility of DNA methylation status associated to prenatal stress is fundamental to contribute to the development of novel specific therapeutic and preventive interventions.

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Chapter 9
Epigenetic Modifications in Borderline Personality Disorder

Reinhard H. Dammann and Gerhard W. Dammann

Abstract Borderline personality disorder (BPD) is a complex psychic disease with an increased importance in the last years. While the diagnosis and therapy are well established, little is known on the pathogenesis of BPD. Epigenetic alterations are a hallmark for abnormal gene expression and could be involved in the etiology of BPD. These epigenetic changes are mainly classified into altered histone modifications and DNA methylation. Here, we summarize recent findings on epigenetic alterations in BPD. Significant aberrant DNA methylation of serotonin receptor 2A (HTR2A), monoamine oxidase A and B (MAOA and MAOB), glucocorticoid receptor (GR/NR3C1), brain derived neurotrophic factor (BDNF), amyloid beta precursor protein-binding family A member 2 and 3 (APBA2 and APBA3), KCNQ1, MCF2, and ninjurin 2 (NINJ2) has been reported. DNA methylation changes range between 1.1- and 1.5-fold increased methylation in the blood of BPD subjects. Especially, GR/NR3C1 methylation was positively associated with childhood maltreatment and clinical severity in BPD. These data may provide new insights into epigenetic mechanisms underlying the pathogenesis of borderline personality disorder.

Keywords Borderline personality disorder • Epigenetics • DNA methylation

9.1 Borderline Personality Disorder

Borderline personality disorder (BPD) is a serious mental disorder characterized by a pervasive pattern of instability in affect regulation, impulse control, interpersonal relationships, and self-image. BPD is discussed to be part of the bipolar (Agius et al. 2012)

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or of the atypical depressive spectrum (Gremaud-Heitz et al. 2014) but less of the schizophrenia spectrum (Dammann 2004).

Clinical signs of BPD include emotional dysregulation, impulsive aggression, repeated self-injury, and chronic suicidal tendencies, which make these patients frequent users of mental health resources (Lieb et al. 2004; Leichsenring et al. 2011). The nine criteria for borderline personality (Diagnostic and Statistical Manual of Mental Disorders: DSM-IV) (APA 1994) can be organized into four sectors of psychopathology: (a) affective disturbance (a range of intense dysphoric affects, sometimes experienced as aversive tension or tremendous mood reactivity), (b) disturbed cognition (overvalued ideas of being bad, experiences of dissociation in terms of depersonalization and derealization, and nondelusional suspiciousness and ideas of reference), (c) impulsivity (e.g., deliberately physically self-destructive and more general forms of impulsivity, disordered eating), and (d) intense unstable relationships (e.g., profound fear of abandonment or tumultuous quality to close relationships). The lifetime prevalence has been estimated to be about 5.9 % (Grant et al. 2008). The point prevalence rates are estimated to be about 0.7 % in European national community samples (Torgersen et al. 2001). Additionally, findings showed that BPD was more common in women than in men (about 70 % and 30 %, respectively) (Widiger and Weissman 1991). The disorder often co-occurs with mood, anxiety, and substance use disorders and is also associated with other personality disorders. The disorder is characterized by severe psychosocial impairment (Skodol et al. 2002a) and a high mortality rate due to suicide – up to 10 % of patients commit suicide, a rate almost 50 times higher than in the general population. Several measures (semi-structural interviews) are highly reliable in the care of these patients (Zimmerman 1994). Borderline personality seems to be less stable over time than expected for personality disorders (Zanarini et al. 2006). Causal factors are only partly known, but genetic factors (Siever et al. 2002; Skodol et al. 2002b; Torgersen et al. 2008) and adverse events during childhood, such as physical and sexual abuse (Soloff et al. 2002), contribute to the development of the disorder.

Etiologies such as trauma, abuse, childhood adversity, and exposure to war appear to influence posttraumatic stress disorder, BPD, emotional dysregulation, empathy, and impulsivity via epigenetic mechanisms (Ripoll et al. 2013). Accumulating evidence points to severe emotional and relationship dysfunction as the core epigenetic expressions of BPD (Steele and Siever 2010). In adulthood, BPD is typified by disorganization within and across interpersonal domains of functioning (Clarkin et al. 2004). When interacting with their infants, mothers with BPD show marked withdrawal and frightening or frightened behavior, leading to disorganized infant-mother attachments. Linked to both infant disorganization and BPD is a maternal state of mind typified by unresolved mourning regarding past loss or trauma. Early risk factors for BPD in adulthood include maternal withdrawal in infancy and separation of 1 month or more from mother in the first 5 years of life (Steele and Siever 2010). The evolutionary based complex gene-environment picture emerging confers risk or protection against BPD pathology in ways consistent with infants varying biological sensitivity to context (Dammann 2003). Maternal separation is influencing epigenetic regulation (Toda et al. 2014).

Epigenetic mechanisms are relevant for the description of the endogenous oxytocin system, playing a central role in social cognition, disturbed in BPD (Puglia et al. 2015). Various forms of epigenetic regulation at the levels of DNA methylation, histone modification, and noncoding RNAs (ncRNAs) can modulate transcriptional and translational events required for memory processes. By changing the cellular profile in the brain's emotional, reward, and memory circuits, these epigenetic modifications have also been linked to perseverant, pathogenic memories typical for posttraumatic stress disorder (PTSD) and BPD (Sillivan et al. 2015). Because epigenetic marks (e.g., DNA methylation) can be modulated by environmental stimuli, DNA methylation may be important in the development of anxiety (Alisch et al. 2014), suicidality (Turecki 2014), and vulnerability for stress (Tsuji et al. 2014) common in BPD.

9.2 Aberrant DNA Methylation in Borderline Personality Disorder

Aberrant epigenetic modifications are hallmarks of altered gene expression and contribute to several diseases including cancer and psychiatric illness (Dammann et al. 2000; Esteller 2007; Jones and Baylin 2007; Tsankova et al. 2007; van Vliet et al. 2007; Abdolmaleky et al. 2008). Epigenetic regulation is mediated through altered chromatin modifications, including changes in DNA methylation levels and aberrant histone marks. Gene promoters are often located in G/C- and CpG-rich DNA sequences which are termed CpG island promoters (Bird 1986). Active CpG island promoters are associated with unmethylated DNA sequences and open chromatin structures. In contrast, inactive promoters are in a repressed chromatin state: CpG sites are hypermethylated and histones are deacetylated. More than 70 % of genes (e.g., housekeeping genes) are transcribed from unmethylated CpG island promoters (Saxonov et al. 2006; Weber et al. 2007). The other genes are transcribed from G/C- and CpG-poor and are rather methylated in the human genome.

For borderline personality disorder, the epigenetic regulation of some disease-associated genes has been investigated in detail (Table 9.1). In a first study, we have analyzed the DNA methylation pattern of 14 neuropsychiatric genes: COMT, DAT1, GABRA1, GNB3, GRIN2B, HTR1B, *5-hydroxytryptamine (serotonin) receptor 2A* (HTR2A), 5-HTT, *monoamine oxidase A* (MAOA), *monoamine oxidase B* (MAOB), NOS1, *glucocorticoid receptor* (GR/NR3C1), TPH1, and TH (Dammann et al. 2011). DNA methylation was analyzed by bisulfite restriction analysis and pyrosequencing in whole blood samples of 26 patients diagnosed with BPD and 11 controls. Aberrant methylation was undetectable by bisulfite restriction analysis. By utilizing pyrosequencing, a significant hypermethylation of HTR2A (1.2-fold), GR/NR3C1 (1.2-fold), MAOB (1.1-fold), MAOB (1.1-fold), and soluble COMT (1.1-fold) was revealed for BPD patients (Table 9.1). Methylation of DAT1, GABRA1, GNB3, GRIN2B, HTR1B, 5-HTT, NOS1, TPH1, and TH was not yet analyzed by pyrosequencing in further details (Dammann et al. 2011).

Table 9.1 Summary of DNA methylation analysis in borderline personality disorder (BPD)

Gene	Methylation increase in BPD compared to controls	Tissue	Control subjects	Methods	References
ABPA3	1.1	Blood	Normal	27 K bead cheap/pyrosequencing	Teschler et al. (2013)
APBA2	1.1	Blood	Normal	27 K bead cheap/pyrosequencing	Teschler et al. (2013)
BDNF (exon I and IV)	2.2 and 3.3, respectively	Blood	52 controls	High resolution melt assay	Perroud et al. (2013)
BDNF (exon IV)	Increased in BPD and bulimia nervosa	Lymphocytes	Non-eating disordered women	Bisulfite and mass spectrometry	Thaler et al. (2014)
DRD2	1.1 in BPD and bulimia nervosa	Lymphocytes	Non-eating disordered women	Bisulfite pyrosequencing	Groleau et al. (2014)
GR/NR3C1 (exon 1_B)	1.2	Blood	Normal	Bisulfite pyrosequencing	Dammann et al. (2011)
GR/NR3C1 (exon 1_F)	1.3 in subjects with childhood maltreatment	Blood	Non-sexually abused	Bisulfite pyrosequencing	Perroud et al. (2011)
GR/NR3C1 (exon 1_C)	1.4-1.5 in BPD with bulimia nervosa	Lymphocytes	Non-eating disordered women or bulimina nervosa (no BPD)	Bisulfite and mass spectrometry	Steiger et al. (2013)
GR/NR3C1 (exon 1_F)	Positive association between methylation and childhood maltreatment	Blood	281 BPD sample	Bisulfite pyrosequencing	Martin-Blanco et al. (2014)
HTR2A	1.2	Blood	Normal	Bisulfite pyrosequencing	Dammann et al. (2011)
KCNQ1	1.5	Blood	Normal	27 K bead cheap/pyrosequencing	Teschler et al. (2013)
MAOA	1.1	Blood	Normal	Bisulfite pyrosequencing	Dammann et al. (2011)
MAOB	1.1	Blood	Normal	Bisulfite pyrosequencing	Dammann et al. (2011)
MCF2	1.1	Blood	Normal	27 K bead cheap/pyrosequencing	Teschler et al. (2013)
NINJ2	1.2	Blood	Normal	27 K bead cheap/pyrosequencing	Teschler et al. (2013)
S-COMT	1.1	Blood	Normal	Bisulfite pyrosequencing	Dammann et al. (2011)

9.3 Aberrant DNA Methylation of GR/NR3C1 and BDNF in BPD

For the neuron-specific glucocorticoid receptor (GR/NR3C1), decreased levels of mRNA as well as increased methylation of the promoter were observed in hippocampus obtained from suicide victims with a history of childhood abuse (McGowan et al. 2009). We and others have reported increased methylation of GR/NR3C1 in borderline personality disorder (Dammann et al. 2011; Perroud et al. 2011; Steiger et al. 2013; Martin-Blanco et al. 2014). In Fig. 9.1, we have indicated CpG sites that exhibited significant changes in methylation levels in these studies. Perroud et al. have analyzed the methylation of exon 1F of GR/NR3C1 in 101 BPD and 99 major depressive disorder (MDD) subjects (Perroud et al. 2011). They report that childhood maltreatment and its severity (e.g., the number of maltreatments) positively correlated with increased NR3C1 methylation (Perroud et al. 2011). For example, they revealed a significant correlation between sexual abuse and GR/NR3C1 methylation showing a 1.34 higher methylation in sexually abused subjects than in nonsexually abused individuals (Table 9.1). These data suggest that early life events may permanently impact the hypothalamic-pituitary-adrenal axis through epigenetic alterations of the GR/NR3C1 (Perroud et al. 2011). Others have analyzed GR/NR3C1 methylation in lymphocytes of women with eating disorder, childhood abuse, or BPD (Steiger et al. 2013). They reported a significant 1.4- to 1.5-fold increased methylation at two out of 18 CpG sites adjacent of exon 1C in women with BPD and bulimia nervosa compared to subjects without BPD (Table 9.1 and Fig. 9.1). No parallel effects owing to childhood abuse were observed (Steiger et al. 2013). Martin-Blanco et al. have analyzed the methylation of GR/NR3C1 (exon 1F) in blood isolated from 281 BPD subjects (Martin-Blanco et al. 2014). They reported a significant positive correlation between GR/NR3C1 methylation and childhood maltreatment (e.g., physical abuse or emotional neglect) (Table 9.1 and Fig. 9.1). Absence of employment was also associated with increased GR/NR3C1 methylation (Martin-Blanco et al. 2014).

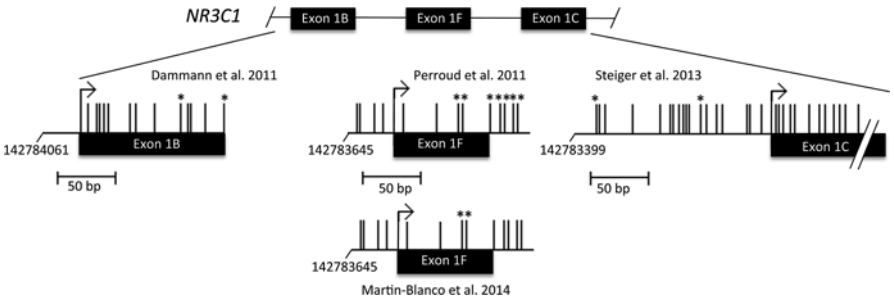

Fig. 9.1 Genomic organization of the analyzed NR3C1/GR exons at chromosome 5q31.3. CpGs are show as *vertical lines* and significant aberrantly methylated CpGs are marked with *asterisks*. Transcription start sites are depicted with *arrows* and genomic positions are indicated. Graphics were generated with the python.vs.cobra program (https://launchpad.net/python.vs.cobra)

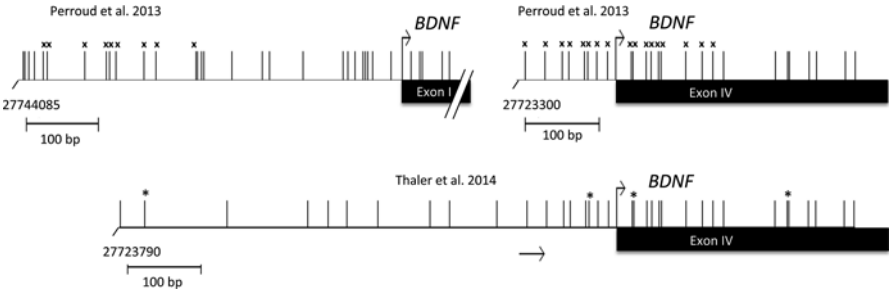

Fig. 9.2 Genomic organization of the analyzed *exon I* and *IV* of *BDNF* at chromosome 11p14.1. CpGs are shown as *vertical lines* and analyzed CpGs are marked with *x*. Thaler et al. (2014) observed that patients with BPD and bulimia nervosa had significant higher methylation levels at four (*asterisks*) out of 27 CpG sites compared to non-eating disorder women. Transcriptions start sites are depicted with *arrows* and genomic positions are indicated. Graphics were generated with the python.vs.cobra program (https://launchpad.net/python.vs.cobra)

Methylation of the brain derived neurotrophic factor (BDNF) gene has also been analyzed in psychiatric disorders, including BPD (Perroud et al. 2013; Mitchelmore and Gede 2014; Thaler et al. 2014). In Fig. 9.2, we have indicated genomic regions of BDNF that were investigated. In peripheral blood cells from 115 BPD subjects and 52 healthy controls, exon I and IV of BDNF were examined for DNA methylation (Perroud et al. 2013). Increased DNA methylation was observed in both exons of BDNF, compared to healthy controls (Table 9.1 and Fig. 9.2). In BPD individuals, a decrease in methylation levels was observed in responders to a 4-week psychotherapy course, whereas nonresponders showed increased methylation (Perroud et al. 2013). Thaler et al. have analyzed the methylation of BDNF in women with bulimic eating syndrome (Thaler et al. 2014). They report that subjects which BPD, eating disorder (bulimia nervosa), or childhood abuse exhibit increased level of methylation at specific CpGs of the exon IV promoter region (Table 9.1 and Fig. 9.2). They observed that patients with BPD and bulimia nervosa had significant higher methylation levels at four out of 27 CpG sites compared to 32 non-eating disorder women (Thaler et al. 2014). Methylation of the dopamine D2 receptor (DRD2) was also analyzed in women with BPD and eating disorder (Groleau et al. 2014). Increased methylation of DRD2 was revealed in eight subjects with BPD and compared to 19 women with no eating disorder and no BPD (Table 9.1). Subjects with eating disorder and without eating disorder did not differ in the mean methylation level of the DRD2 promoter (Groleau et al. 2014).

In summary, these data suggest that aberrant methylation of disease-relevant genes (e.g., GC/NR3C1 and BDNF) occurs in peripheral blood of BPD patients, and these methylation marks may serve as candidate tool to identify potentially affected BPD subjects.

9.4 Epigenetic Alterations Detected by High Throughput Technology in Borderline Personality Disorder

Microarray (chip) technology is a high throughput assay to identify disease-related candidate genes. For microarray-based methylation analysis, two different methylation specific-bead chips from Illumina are available: the HumanMethylation27k bead chip (>27,000 CpG sites) and the second generation HumanMethylation450k bead chip (>450,000 CpG sites) (Bibikova et al. 2006; Sandoval et al. 2011). We have performed 27k methylation bead chip assay with bisulfite converted DNA from blood of 26 BPD patients and 11 controls (Teschler et al. 2013). As expected, our results show that no huge difference in methylation levels between BPD patients and controls are found (Teschler et al. 2013). Genome wide methylation levels of blood samples of BPD patients and control samples are rather similar. However, on several gene-specific CpG sites, bead chip technology and quantitative bisulfite pyrosequencing showed a significantly increased methylation of amyloid beta (A4) precursor protein-binding family A member 2 and 3 (*APBA2:* 1.1-fold and *APBA3:* 1.1-fold), potassium voltage-gated channel KQT-like subfamily member 1 (*KCNQ1:* 1.5-fold), MCF.2 cell line derived transforming sequence (*MCF2*: 1.1-fold), and ninjurin 2 (*NINJ2;* 1.2-fold) in BPD patients (Table 9.1). For the CpG sites of *GATA4* and *HLCS,* an increase in DNA methylation was observed but was only significant in the bead chip assay (Teschler et al. 2013). These results show a significant 1.26-fold average increase in methylation at the analyzed gene-associated CpG sites in the blood of BPD patients compared to controls (Teschler et al. 2013). It is interesting to note that three of these genes (*APBA2*, *APBA3,* and *NINJ2*) are correlated with Alzheimer's disease.

Others have utilized the HumanMethylation450k bead chip to analyze methylation level in DNA isolated from peripheral blood leukocytes in 96 BPD subjects suffering from a high level of child adversity and 93 subjects with major depressive disorder (MDD) and a low rate of child maltreatment (Prados et al. 2015). They report significant differential methylation of IL17RA, miR-124-3, KJCNQ2, EFNB1, OCA2, MFAP2, RPH3AL, WDR60, CST9L, EP400, A2ML1, NT5DC2, FAM163, and SPSB2 either in BPD compared with MDD or in relation to the severity of childhood maltreatment (Prados et al. 2015). Aberrant methylation of one CpG site of miR124-3 was also analyzed by pyrosequencing in BPD and MDD subjects; however, differential methylation was insignificant by this assay (Prados et al. 2015). The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing and could be involved in the regulation of GR/NR3C1 (Makeyev et al. 2007; Prados et al. 2015).

9.5 Altered Histone Modifications in Borderline Personality Disorder

Aberrant histone modifications represent another important epigenetic alteration that may cause changes in expression pattern of disease-related genes in borderline personality disorder. However, histone modifications were not investigated in BPD up to now. The main method to analyze histone modifications or histone variants is cross-linking chromatin in tissue samples and to utilize specific antibodies to precipitate and to quantify chromatin changes. For obvious reasons, relevant fresh tissue samples are rather difficult to obtain from human subjects. Candidate genes to investigate of aberrant histone modification or variants are BDNF, HTR2A, and GR/NR3C1 (Perroud et al. 2011; Mitchelmore and Gede 2014; Paquette and Marsit 2014).

9.6 Conclusions

Increased DNA methylation of several neuropsychiatric genes occurred in blood of BPD patients and was also linked to child abuse or clinical severity (e.g., GR/NR3C1) (Perroud et al. 2011; Martin-Blanco et al. 2014). A number of studies have suggested a role for environmentally mediated aversive events in the development of BPD and have found an association between the diagnosis of BPD and psychotraumatization during childhood (Herman et al. 1989; Famularo et al. 1991; Goldman et al. 1992; Paris et al. 1994; Waller 1994; Silk et al. 1995). In a family environment, early maltreatment (childhood abuse and neglect) within a family environment may be particularly important in producing long-term epigenetic changes (Gunnar and Quevedo 2007). In animal models, postnatal maternal care has been linked to epigenetic alteration via DNA methylation (Kaffman and Meaney 2007; McGowan et al. 2011). In the blood of BPD subject, aberrant methylation of several neuropsychiatric genes was revealed, and these findings are summarized in Table 9.1. In future work, it will be interesting to verify this methylation changes in other tissues and to reveal novel BPD-specific biomarkers.

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Chapter 10
Oxytocin Receptors and Neurobehavior

Robert Kumsta, Johannes C.S. Zang, and Dirk Moser

Abstract Research across species has shown that the neuropeptide oxytocin plays a key role in the regulation of social cognition and behavior. It is important for attachment, social exploration, and social recognition, as well as anxiety and stress-related behaviors. Based on oxytocin administration studies and measurements of peripheral oxytocin levels, it has been suggested that signaling of oxytocin is impaired in mental disorders associated with social deficits, including autism, borderline personality disorder, and social anxiety disorder. There are several factors influencing interindividual differences in social-cognitive abilities and differences in the susceptibility to psychiatric disorders, including variability in genes involved in oxytocin signaling. In addition to sequence variation, interindividual differences might in part be explained by variation in epigenetic processes influencing gene expression. Here, we provide an overview of the functional organization and epigenetic regulation of the murine and human oxytocin receptor gene.

Studies in mice have shown that the oxytocin receptor gene (*Oxtr*) is epigenetically regulated by DNA methylation with experience- and tissue-specific expression patterns. In humans, functional studies on epigenetic mechanisms have focused on oxytocin receptor gene (*OXTR*) DNA methylation and have provided evidence for the influence of *OXTR* promoter methylation on *OXTR* mRNA expression. A small number of studies have investigated the role of *OXTR* methylation in behavioral phenotypes and mental illness. There is first evidence that *OXTR* methylation is associated with different aspects of social cognition as well as with psychiatric disorders characterized by deficits in social cognition, including autism, high callous-unemotional traits in youth, social anxiety, and anorexia nervosa.

Given evidence that epigenetic states of genes can be modified by experiences, especially those occurring in sensitive periods early in development, we conclude with a discussion on the effects of traumatic experience on the developing oxytocin system. Epigenetic modification of genes involved in oxytocin signaling might play a part in the mechanisms mediating the long-term influence of early adverse experiences on socio-behavioral outcomes.

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10.1 Functional Organization and Epigenetic Regulation of the Oxytocin Receptor Gene in Mice and Human

*10.1.1 The Murine Oxytocin Receptor Gene (*Oxtr*)*

Several rodent species, including voles, rats, and mice, have been used as model organisms to characterize the role of the oxytocin in social behaviors. However, in terms of epigenetic regulation, only the murine *Oxtr* has been investigated systematically so far. The murine *Oxtr* gene sequence was first published by Kubota and colleagues (1996) who mapped it to chromosome 6 (D86599; chr6: 112.425.884-112.440.884; July 2007 – NCBI37/mm9). It covers a region of about 20 kilobases (kb) and is comprised of four exons and three introns. The start codon of the 2914 nucleotide mRNA transcript is located within exon III, with a coding sequence extending into exon IV as matrix for a 388 amino acid (aa) seven transmembrane domain receptor. At the DNA sequence level, the murine *Oxtr* displays 87 % similarity to the coding sequence of its human orthologue; on protein level, 92 % similarity between murine and human oxytocin receptor is found.

In its 5′ regulatory region, the *Oxtr* promoter lacks typical elements of eukaryotic promotors like a TATA or CAAT box but contains an 859 basepair (bp) CpG island[1] (CGI), including 89 CpG sites (surrounding exon 3). Furthermore, the *Oxtr* 5′ regulatory region harbors multiple transcription-factor-binding sites (TFBS) including recognition motifs for Nfκb, Creb, Sp1, Nf, and Il6, as well as several half-palindromic estrogen receptor elements (half EREs), and one palindromic ERE, which is located in close proximity to the transcription start site (TSS; Kubota et al. 1996).

10.1.2 Regulation of Oxtr *Expression by DNA Methylation*

Temporal and spatial Oxtr expression patterns are associated with a number of factors, e.g., labor and parturition (Gould and Zingg 2003), as well as fluctuations in levels of steroid hormones, such as estrogen (Ivell and Walther 1999; Fleming et al. 2006). To what extent promoter CpG methylation might contribute to time and tissue-specific differences of *Oxtr* expression has not been studied in great detail so

[1] Certain areas of the genome contain regions of high CpG density. These regions, called "CpG islands," are defined as a >200 bp region with GC content of more than 50 % and an observed/predicted CpG ratio of more than 0.6 (Gardiner-Garden and Frommer 1987). CpG islands often span the promoter region of genes and are associated with active gene expression (Saxonov et al. 2006). These stretches of DNA have a higher CpG density than the rest of the genome and tend to be unmethylated (Bird et al. 1985). However, when methylated, CpG islands in gene promoters contribute to transcriptional repression in most tissues (Razin 1998).

far. Mamrut and colleagues (2013) investigated the influence of single CpG sites on the *Oxtr* promoter activity in vitro. Working with mouse hypothalamic cell lines, the authors designed a reporter vector system under control of a 1463 bp *Oxtr* minimal promotor sequence (−1417/+46). Their analysis focussed on the effects of DNA methylation within a 415 bp region, containing seven CpGs, of which the first CpG is located within a half-ERE and the last resides in a Sp1 binding site. In addition to the original reporter construct, they also created three modified versions by site directed mutagenesis of two CpGs located in these TF-binding motifs and by deletion of the 415 bp fragment comprising all seven CpGs (Fig. 10.1c). The influence of CpG methylation on *Oxtr* expression was assessed by comparing the different promoter efficiencies of the methylated or unmethylated constructs. Results showed that methylation of two CpG sites (one in the half-ERE, one in the Sp1 binding site) are mostly responsible for the overall effect of *Oxtr* promoter methylation on gene expression. Based on their findings, the authors propose a model of *Oxtr* regulation which is mediated by Sp1 and estrogen receptor signal transduction processes in

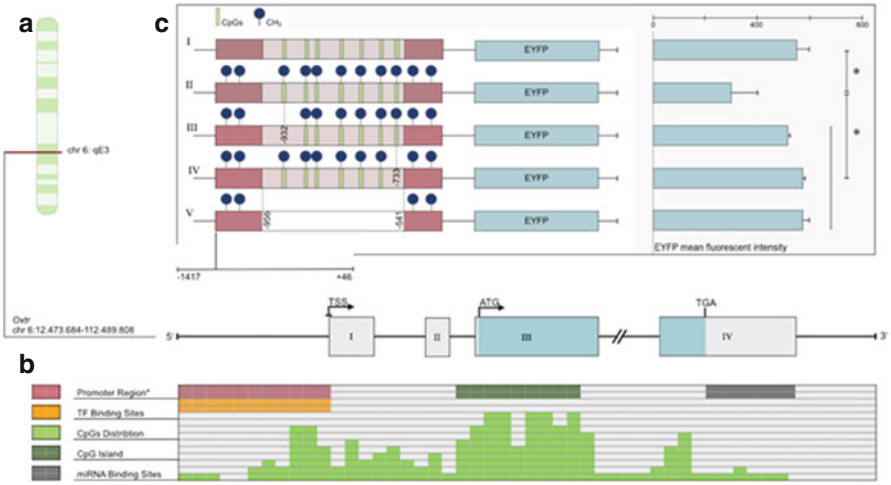

Fig. 10.1 Panel (**a**) shows the location of the murine *Oxtr* on chromosome 6: qE3. The *Oxtr* gene sequence covers a region of ~20 kilobases (kb). It is comprised of four exons, depicted as *gray boxes* (*I–IV*), and three introns. The coding region is shaded in *blue* and resides within Exon *III* and *IV. Arrows* indicate the transcription start site (TSS) and ATG start codon. Panel (**b**) shows the relative location of the core-promotor-region in *red* (as described by Mamrut et al. 2013). The area with a high frequency of transcription factor (TF) binding sites is shown in *orange*, and the location of the CpG island is depicted by *dark green*, and the relative distribution of CpG sites in *lighter green. Higher bars* represent higher CpG content. Potential micro RNA binding sites as predicted by Target Scan are shown in *light blue*. The Influence of site-specific absence of methylation in the promotor region (−1417/+46) on gene expression is depicted in panel (**c**). The different constructs are numbered by *roman numerals*. Within the constructs, promotor regions are outlined by *red boxes*, the eYFP reporter genes by *blue boxes* and the analyzed region containing the Sp1 an ERE binding sites is shown in *lighter shade*. CpGs are shown by *green bars*, and methyl groups are depicted by *black circles. Numbers* indicate the location of CpGs abolished by C to A mutation (*III* and *IV*) or the deleted region (*V*). *Blue bars* on a *gray background* indicate the relative fluorescent activity, and *asterisks* indicate significant differences. The fully methylated construct (*II*) is associated with lowest transcriptional efficiency. Deletion of the CpG in the ERE (*III*) or in the Sp1 site (*IV*) leads to a rescue in transcriptional efficiency. See text for further details

interaction with the methylation status of the corresponding transcription factor binding sites. These results highlight that methylation of single CpG sites can have significant functional effects. It is of note that no promoter activity is predicted by bioinformatics analysis (bimas promoterscan software; Prestridge 1995) for the region investigated here. However, high promoter activity is predicted for a region located ~2 kb downstream and surrounding the CpG island and *Oxtr* exon II and III as initially described by Kubota et al. (1996). More detailed investigation of *Oxtr* promoter activity is warranted to get a full picture of the effects of promoter methylation on *Oxtr* regulation patterns.

Mamrut and colleagues (2013) further showed that *Oxtr* promoter methylation is modified in a tissue-specific manner under different conditions in vivo. While 1 h after parturition *Oxtr* promoter methylation increased in the mammary glands, it decreased in the uterus. Parturition seemed to specifically influence site-specific methylation of five CpGs within the analyzed promotor region. Twenty-four hours after parturition, the mean methylation levels of these CpGs were found to have returned to the baseline level in the uterus. In contrast, they stayed elevated in the mammary gland. Surprisingly, Mamrut and colleagues (2013) reported a positive relationship between the averaged methylation score across five CpGs and quantified *Oxtr* mRNA levels in the mammary gland and a negative one in the uterus.

In a follow-up study, Harony-Nicolas et al. (2014) investigated *Oxtr* promoter methylation and the corresponding mRNA expression in various regions of the mouse brain (anterior olfactory nucleus, caudate putamen, cerebellum, cerebral cortex, medial amygdala, olfactory bulb, and ventromedial hypothalamus). Differential methylation of *Oxtr* promoter was observed between these brain regions; however, only methylation of the CpG site located in the SP1 binding sequence was correlated (positively) with *Oxtr* mRNA expression.

So far, only these two studies have systematically investigated the effects of DNA methylation on Oxtr gene regulation. The emerging picture is that DNA methylation of the *Oxtr* promoter is functionally involved in the gene's regulation. The observed tissue-specific differences, as well as the suggestion of DNA methylation changes over time in the context of parturition, and not least the observation that *Oxtr* promoter methylation is both correlated with decreased and increased *Oxtr* mRNA expression highlight the complexity surrounding epigenetic regulation of *Oxtr* expression. Future studies involving other gene regulatory regions outside the core promoter are warranted. Furthermore, it will be crucial to further elucidate not only the association between *Oxtr* methylation and expression levels over tissues and time but also to determine whether those predict the amount of actual functional protein in a given cell type.

*10.1.3 The Human Oxytocin Receptor Gene (***OXTR***)*

The human oxytocin receptor gene on chromosome 3 (NM_000916; chr3: 8792095-8811300; Feb. 2009 – GRCh37/hg19) is controlled by a promoter containing a 2319 bp CpG island with 188 CpG sites, which stretches from −20 bp to +2350 bp

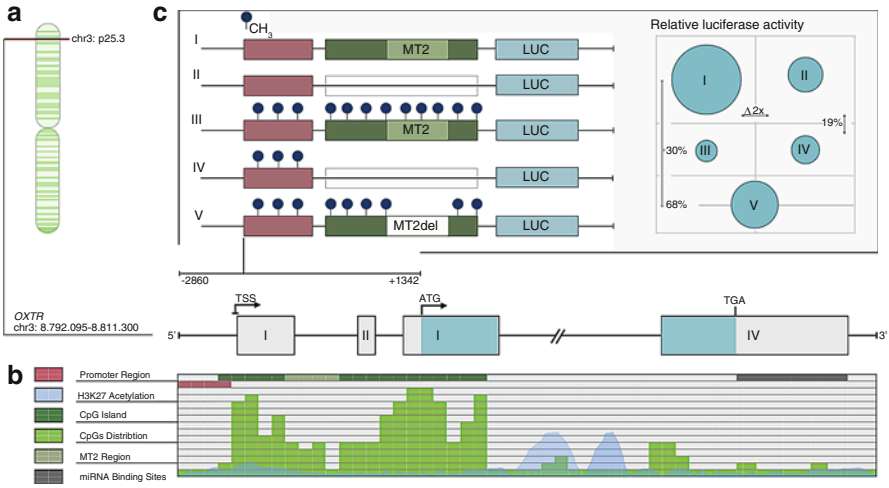

Fig. 10.2 Panel (**a**) shows the location of the human *OXTR* on chromosome 3:p25.3. Its sequence covers a region of about 20 kb. It is comprised of four exons, depicted as *gray boxes* (*I–IV*), and three introns. The coding region is shaded in *blue* and resides within exon *III* and *IV. Arrows* indicate the transcription start site (TSS) and ATG start codon. Panel (**b**) shows the relative location of the core-promotor-region (*red*) and the regions characterized by frequent H3K27-acetylation (*light blue*). The CpG island is shown in *dark green*, the MT2 region within the CpG island is depicted in *lighter green*. The relative distribution of CpG sites is indicated in *green*, with *higher bars* representing higher CpG content. Panel (**c**) shows the effects of methylation in the CpG island on gene expression (Kusui et al. 2001). The different constructs are numbered by *roman numerals*. Within the constructs, promotor regions are outlined by *red boxes*, the Luciferase reporter genes by *blue boxes*. The CpG island is depicted by *dark-green boxes*, and the MT 2 region is shown in *light green*. Methyl groups are indicated by *dark blue circles. Light blue* circles on a *gray background* indicate the relative luciferase activity. See text for details

with reference to the transcription start site (Fig. 10.2a). It controls the expression of a 4361 nucleotides mRNA transcript, which is translated into a 389 aa protein. Kusui and colleagues (Kusui et al. 2001) investigated whether methylation of the *OXTR* CpG island influenced *OXTR* transcription. Using a luciferase reporter gene assay, it was shown that the CGI has significant promoter activity. Transcriptional activity of an unmethylated reporter construct including the CpG island (−2860 bp to +1342 bp relative to the transcription start site) was about 2-fold higher compared to the construct including the core promoter but lacking the majority of the CpG island (−2860 bp to +144 bp). Following methylation, transcriptional activity of the reporter gene construct lacking the CGI was reduced by 19 %, whereas activity of the construct including the CpG island was suppressed by 68 % when methylated (Fig. 10.2c). This indicates that *OXTR* CGI methylation functionally suppresses transcription, at least in the investigated hepatoblastoma cell line.

Importantly, Kusui et al. identified a region of the *OXTR* CGI (termed MT2; see Fig. 10.2c) that appears to be responsible for the majority of DNA methylation-induced silencing of these constructs. Deletion of the MT2 region in the full length construct led to a relative rescue of transcriptional activity of the methylated construct to 68 %. This suggests that regulation of *OXTR* is sensitive to methylation

within the MT2 region of the CGI and points towards functional significance of this region. However, the precise mechanism of how increased methylation in CpG sites of the MT2 region leads to transcriptional downregulation of *OXTR*, and how this relates to receptor distribution in target tissue, is currently unknown and should be addressed in postmortem investigation of brain tissue derived from healthy samples as well as patient groups.

10.2 *OXTR* Methylation and Its Association with Social-cognitive and Psychiatric Phenotypes in Humans

10.2.1 Social Perception

In addition to its role in social cognition and affiliative behavior, oxytocin seems to be involved in the processing of basic social stimuli. For instance, intranasal oxytocin administration improved emotion recognition (Domes et al. 2007b, 2010), increased covert attention to positive social cues (Domes et al. 2012), and time spent looking at the eye region of faces (Guastella et al. 2008).

Building on evidence of *OXTR* hypermethylation and related transcriptional differences in the temporal cortex of autistic individuals, Jack et al. (2012) investigated whether methylation of a particular CpG site (−934) might be related to activation differences in brain regions involved in biological motion perception. Functional MRI data were collected while participants ($n = 42$) passively viewed a scene in which geometrical shapes interact in ways suggestive of animacy.

Whole-brain analysis showed that individuals with higher levels of *OXTR* methylation in PBMCs demonstrated significantly greater activation in two clusters. The first extended from the superior temporal gyrus into supramarginal gyrus at the temporal parietal junction, and the second was located in the dorsal anterior cingulate cortex (dACC). The temporal parietal junction in particular has been linked to the attributions of intentions, perception of biological motion cues, and mentalizing behaviors (Blakemore et al. 2003). These results suggest that *OXTR* methylation influences relatively low-level processes involved in social perception and may contribute more generally to interindividual differences in social cognition and behavior.

In a follow-up study (Puglia et al. 2015), the association between methylation of the same CpG site with neural responses during emotional face processing was investigated in a gender-balanced sample of 98 healthy participants. *OXTR* methylation level at this site showed high inter-subject variability and ranged from 33 % to 72 % methylation. Region of interest analyses focussed on the amygdala and showed that increased methylation levels predicted increased BOLD activity in left amygdala when participants viewed angry and fearful faces. In a set of exploratory analyses, it was shown that increased levels of methylation predicted increased BOLD activity in multiple brain regions, including areas associated with emotion regulation

such as insular cortex, and dACC, as well as regions associated with social perception (fusiform gyrus and posterior superior temporal sulcus). Furthermore, investigation of task-specific amygdala functional connectivity revealed that connectivity between neural systems supporting social perception was attenuated by *OXTR* methylation. Higher methylation levels were related to reduced right amygdala functional connectivity with multiple brain regions, such as insular cortex, cingulate cortex, and orbitofrontal cortex, as well as regions associated with face perception, such as fusiform gyrus.

These two studies show that *OXTR* methylation levels at a single CpG located in a functionally relevant gene region predict neural responses and functional connectivity within regions supporting biological motion perception, social perception, and emotion processing. Future studies should extend methylation analyses beyond the one site studied here, and study the effects of methylation on social perception in naturalistic settings.

10.2.2 Psychosocial Stress

In addition to its role in social behavior and social cognition, oxytocin influences the mammalian physiological stress response. It interacts both with the hypothalamus pituitary adrenal (HPA) axis (Neumann 2002) and with sympathetic nervous system stress reactivity (Ditzen et al. 2013). In combination with social support, intranasal administration of oxytocin has been shown to dampen neuroendocrine stress reactivity (Heinrichs et al. 2003) and to decrease amygdala activation in response to threatening stimuli (Kirsch et al. 2005; Domes et al. 2007a). Neurogenetic studies provide further evidence for the involvement of oxytocin signaling in stress reactivity (Chen et al. 2011).

Unternährer et al. (2012) investigated whether dynamic changes in *OXTR* DNA methylation might be observed after acute stress exposure. A sample of 76 participants was subjected to the Trier Social Stress Test (TSST), a standardized laboratory protocol consisting of extemporaneous public speaking and mental arithmetic tasks. Methylation levels of 35 CpG sites, located mainly in the protein coding part of exon 3 (see Fig. 10.2), were assessed immediately before, 1 min after, and 90 min after stress exposure. Mean methylation status increased immediately after stress and then decreased to below baseline levels 90 min poststress.

While this investigation provides first evidence that DNA methylation status of a gene involved in stress regulation might be sensitive to acute stress exposure, these results should nevertheless be interpreted with caution. First, methylation was assessed in whole blood. As the composition of the circulating leukocyte pool can rapidly change in response to stress (Richlin et al. 2004), changes in blood cell composition might partially account for the observed differences in methylation levels. Second, the observed differences were rather small. The mean change from pre- to poststress was 0.38 %, and 1.04 % from poststress to follow-up, although individual CpGs showed larger differences.

The notion of rapid changes in methylation following stress exposure is intriguing. However, in order to further investigate potential underlying mechanisms, replication studies should also include measures that might help to specify which components of the stress signaling cascades might be involved, i.e., glucocorticoid or catecholaminergic signaling, or both.

10.2.3 Autism

Several lines of evidence have implicated the oxytocin system in the etiology of autism spectrum disorder (Heinrichs et al. 2009). Whereas a number of studies have shown associations between variants of *OXTR* and autism spectrum disorder (Ebstein et al. 2012), we are aware of only one investigation assessing the role of differential methylation of *OXTR* in autism. The starting point for the study by Gregory et al. (2009) was the identification of an allelic deletion of *OXTR* in an autistic boy and his mother. The boy's affected brother, although not inheriting the deletion, showed increased methylation at two CpGs located in the MT2 region compared to the nonautistic father. This raised the possibility that methylation of *OXTR*, as a mechanism of epigenetic silencing, might be involved more generally in the etiology of autism. In a next step, analyses were extended beyond this family, and *OXTR* methylation in DNA from PBMCs was investigated in 20 individuals with autism and 20 matched normotypical controls. Significantly increased methylation in autistic probands was observed for CpGs −860, −934, and −959 (numbering according to translation start site +1). Importantly, a similar pattern of methylation differences was observed in temporal cortex tissue. In an independent sample of 10 autistic and matched controls, increased methylation of CpGs −860, −901, −924, and −934, with correspondingly reduced expression of *OXTR* mRNA by 20 %, was observed. Although investigated in a small sample, these findings suggest functional importance of *OXTR* promoter methylation with regard to gene regulation and possibly the etiology of autism.

10.2.4 Social Anxiety

Social anxiety disorder (SAD) is among the mental disorders characterized by social dysfunctioning. It has been hypothesized that oxytocin dysregulation might be implicated in the etiology of SAD, as decreased baseline oxytocin plasma levels have been observed in patients (Hoge et al. 2008), and intranasal application of oxytocin in the context of exposure therapy improved self-reported speech performance compared with placebo (Guastella et al. 2009).

In a multilevel study, Ziegler et al. (2015) investigated in 110 unmedicated patients with SAD and 110 unaffected controls whether *OXTR* methylation in peripheral blood was associated with the categorical phenotype of SAD and with

dimensional traits related to social anxiety in both patients and healthy probands. Furthermore, the association between *OXTR* methylation and cortisol responses to psychosocial stress was investigated in a subsample of healthy participants ($n = 16$). Lastly, brain activation patterns during processing of social phobia-related words were investigated in SAD patients ($n = 25$).

Categorical analyses showed that out of 12 investigated CpGs in the coding region of the gene (exon 3, see Fig. 10.2), 7 were differentially methylated between patients and controls. Six were relatively hypomethylated in SAD patients, with a mean difference of 2.4 % (range 0.1–9 %). One of the CpGs was slightly higher methylated by 1.1 % in the SAD patients.

Dimensional analyses revealed a negative correlation between mean *OXTR* methylation and two self-report measures of social anxiety severity (*Social Phobia Scale* and *Social Interaction Anxiety Scale*). Analyses of the intermediate phenotypes of social anxiety revealed: (i) a negative correlation of mean *OXTR* methylation levels and maximum cortisol response to the TSST, and, (ii) a negative correlation of average *OXTR* methylation across all 12 sites and amygdala responsiveness to social phobia-related words contrasted with generally negative words.

These results provide first evidence that methylation of *OXTR* might be associated with SAD as well as with social phobia-related dimensional measures. It is, however, unclear how relative *OXTR* hypomethylation might be mechanistically linked to SAD, especially since the functional consequences of DNA methylation in the investigated region (exon 3) of the gene have not been investigated yet. Furthermore, it is unknown whether small differences in methylation are biologically meaningful at all. The authors speculate that these findings might reflect a compensatory mechanism leading to an upregulation of oxytocin receptor expression against the background of low oxytocin levels.

10.2.5 Callous-Unemotional Traits

Mostly on theoretical grounds, researchers have argued for a role of oxytocin in psychopathy (Moul et al. 2012) and callous-unemotional (CU) traits, considered a developmental precursor to psychopathy (Frick and White 2008). Psychological processes that are disturbed in psychopathy, i.e., emotion recognition, empathy, and social affiliation, are all influenced by oxytocin signaling. However, apart from a few candidate gene studies associating *OXTR* SNPs with conduct problems or CU traits (Beitchman et al. 2012; Sakai et al. 2012), empirical evidence is scarce. Dadds et al. (2014) provided first evidence that *OXTR* promoter methylation is associated with CU traits in males. A subsample of 69 boys (3–16 years old) who met formal criteria for DSM-IV diagnosis of conduct problems (Oppositional Defiant Disorder or Conduct Disorder) was investigated. DNA was extracted from blood cells, and methylation levels across 5 CpG sites (−989, −959, −934, −924, −826) located in the MT2 area were assessed and combined into a mean methylation score. Overall, there was no significant association between methylation and CU traits. However,

when taking into account the age, a different pattern emerged. Whereas there was no relation between methylation and CU traits in the younger sample (3–8 years), higher methylation of *OXTR* was associated with elevated CU traits in the older sample (9–16 years).

The association between *OXTR* methylation and CU traits observed in this cross-sectional study was replicated in the Avon Longitudinal Study of Parents and Children (ALSPAC), a prospective-longitudinal study of children now aged about 24 years. The investigated subsample of the ALSPAC cohort consisted of 84 individuals with early-onset and persistent conduct problems (Cecil et al. 2014). DNA was extracted at birth (cord blood), age 7, and age 9 years (both peripheral blood). DNA methylation was assessed genome-wide (Illumina HumanMethylation450K BeadChip Array), and 12 CpG sites located within the *OXTR* CGI were analyzed. The analyses focussed on 3 CpG sites located in the coding region of exon 3 (the remaining CpG sites were either not associated with CU traits or showed no variably in methylation level). Results provided evidence for distinct etiological pathways to CU. *OXTR* methylation at birth was prospectively associated with CU traits at age 13 years, but only for youth with low internalizing symptoms. Interestingly, in the same group, higher prenatal parental risks such as maternal psychopathology, criminal behaviors, and substance use associated with higher *OXTR* methylation at birth. *OXTR* methylation at birth was also associated with reduced experience of direct victimization by peers and adults, a finding the authors refer to as an "evocative epigenetic–environment correlation." Furthermore, *OXTR* methylation had greater temporal stability for the group with low internalizing symptoms. The present study supports the existence of distinct developmental pathways to CU and links prenatal risk factors to high CU traits in early adolescence via *OXTR* methylation at birth.

10.2.6 Eating Disorder

There is suggestive evidence of abnormal oxytocin functioning in patients with anorexia nervosa (AN). Altered cerebrospinal fluid (CSF) and serum oxytocin levels have been observed in AN patients (Maguire et al. 2013), and oxytocin levels were associated with brain activation patterns in response to images of food (Lawson et al. 2012). DNA methylation of a small number of candidate genes has been investigated in AN, with mixed results. Kim et al. (2014) investigated DNA methylation of the *OXTR* derived from buccal cells in 15 female patients with AN and 36 female healthy controls. They analyzed methylation status of 49 CpG sites in a region of about 600 bp in size that includes the untranslated first exon and the MT2 region. Out of the 49 CpG sites examined, six showed significant differences in methylation between AN patients and the control group, with five sites displaying higher methylation levels in AN patients. For the five sites with higher methylation in the AN group, the differences in methylation level ranged from 28 % to 60 %, which represent remarkably large differences. Four of these CpGs fall within the MT2 region,

making it likely that methylation differences might influence *OXTR* gene regulation (see above). Furthermore, individual methylation levels at all CpG sites with higher methylation levels in the AN group correlated negatively with BMI across all participants (correlation coefficients ranged between –0.35 and –0.70).

It is unclear whether differences in *OXTR* DNA methylation reflect a risk factor for AN, possibly due to environmental influences in early development, or whether they are a secondary consequence of the illness. Since nutrients and bioactive food components are known to influence epigenetic processes, AN related factors such as weight loss and changes in dietary composition, including changes in folate availability, may have contributed to the increased methylation (Yi et al. 2000; Choi et al. 2013b).

10.3 The Oxytocin System as a Potential Target for Developmental Programming

There is growing evidence that epigenetic states of genes can be modified by experiences, especially those occurring in sensitive periods early in development. In their seminal studies on rodents, Michael Meaney and his colleagues demonstrated a functional link between naturally occurring variations in maternal behavior and specific epigenetic modifications leading to changes in gene expression and lifelong phenotypic differences in physiology and behavior, including neuroendocrine stress responsivity, fear-related behavior and attentional processes, synaptogenesis, and cognitive development (for review see Zhang and Meaney 2010). In the context of this chapter (but see also Vol 1, Part I, Chap. 2; Vol 1, Part II, Chap. 9), the influence of maternal rearing on maternal care itself is of relevance. Offspring of rat mothers who display a low frequency of pup licking/grooming (LG) have reduced oxytocin receptor binding during the postpartum period (Champagne et al. 2001). There is no evidence of differential methylation related to variations in maternal care of the *Oxtr* itself. However, it is likely that estrogen–oxytocin interactions serve as a mechanism for the differences in oxytocin receptor binding and maternal behavior between lactating high and how LG females. High levels of maternal care received in infancy are associated with decreased Stat5 binding site methylation in the *estrogen receptor 1* (*Esr1; Erα*) *p*romoter and increased levels of Esr1 in the medial preoptic area (MPOA; Champagne et al. 2006). This serves to increase estrogen sensitivity in response to the rising hormone levels experienced in late gestation increasing oxytocin receptor binding. Potentially, this might lead to activation of mesolimbic dopaminergic neurons, which serve to increase the duration and frequency of LG provided towards pups. Through these pathways there may be a behavioral transmission of oxytocin related behavior from mother to offspring through epigenetic modification of *Esr1* (Champagne 2008; Peña et al. 2013).

To our knowledge, there is no published research in humans on the effects of early environmental influences on differential *OXTR* methylation. However, there is evidence that the developing central nervous oxytocin system is affected by early

adversity. In a sample of adult women with a history of early abuse, decreased oxytocin concentrations in cerebrospinal fluid (CSF) were found in women reporting exposure to childhood abuse as compared to women without such experience (Heim et al. 2008b). Prolonged institutional deprivation in early childhood also seems to interfere with the developing oxytocin system. Changes in oxytocin levels after social interaction were investigated in postinstitutionalized children reared in severely depriving conditions (Wismer Fries et al. 2005). Compared to children reared in a typical home environment, the adopted children showed lower peripheral oxytocin levels after physical interactions with their adoptive mothers.

It has been hypothesized that the observed long-term effects of adverse childhood experiences on deficits in social behavior and cognition might be mediated through oxytocin functioning (Heim et al. 2008a). The observation that the effects of adverse childhood experiences last well beyond childhood and increase the risk for the development of a wide variety of diseases in adulthood (Gilbert et al. 2009) points towards enduring biological effects underlying these associations and also raises the question of how these effects retain their stability. Epigenetic mechanisms potentially constitute such a mechanism, serving as a molecular link between "nurture" and "nature." Future studies are warranted to investigate the role of epigenetic regulation of genes involved in oxytocin signaling in mediating the long-term influence of early adverse experiences on socio-behavioral outcomes.

10.4 Beyond DNA Methylation

Most epigenetic studies, at least in humans, focus on DNA methylation patterns and their association with phenotypes of interest. There are, of course, other mechanisms involved in the control of gene regulation, such as histone modification and control of mRNA expression by noncoding RNAs, especially miRNAs (see below). In the context of oxytocin signaling, an epigenetic mechanism underlying pair bond formation in prairie voles involving histone modifications has been described. Several neurotransmitters, including oxytocin, vasopressin, and dopamine, regulate partner preference formation in the socially monogamous prairie vole. An epigenetic basis in partner preference formation was hypothesized, as variations in gene expression of *Oxtr* and *Avpr1a* are known to influence partner preference (Ross et al. 2009; Keebaugh and Young 2011), and as histone deacetylase (HDAC) inhibitors, which enhance gene expression through increased histone acetylation can directly influence social behavior such as sexual receptivity (Bonthuis et al. 2011). Wang et al. (2013) could show that HDAC inhibitor (HADCi) administration in the *nucleus accumbens* (NAcc) induced partner preference in the absence of mating and led to higher levels of mRNA and protein expression of oxytocin receptor in the NAcc. This mimicked the naturally occurring processes of pair bonding, as cohabitation and mating with a male increased *Oxtr* and *Avpr1a* expression through enhanced histone acetylation in the NAcc. Thus, during cohabitation with a male, HDAC inhibition with trichostatin A (TSA) or mating rapidly induces a specific

histone H3 acetylation at the *Oxtr* promoter in the NAcc. This leads to enhanced transcription, resulting in higher oxytocin receptor mRNA and protein expression, which thereafter facilitates partner preference formation. Importantly, HDAC inhibition alone could not induce the partner preference; rather, it facilitated the formation of partner preference through the potentiation of naturally occurring neuronal processes triggered by cohabitation with a male. Such potentiation has also been observed in the context of memory formation and extinction learning and has been referred to as "epigenetic priming" by HDACi (Stefanko et al. 2009; Hawk et al. 2011; Graff and Tsai 2013; Graff et al. 2014). Taken together, this study shows that transient epigenetic changes involving histone modifications of the *Oxtr* promoter are necessary for the long-term change in social behavior.

10.5 Summary

Differential methylation of regulatory elements in both the murine and the human oxytocin receptor gene seem to be functionally important for the gene's expression. In humans, differences in the degree of DNA methylation have been observed in childhood disorders characterized by impairments in social cognition. Furthermore, differential methylation of oxytocin receptor gene might be important in explaining individual differences in social behavior and cognition more broadly and might provide a mechanism for biological embedding of early experience. Potentially, improved epigenetic understanding of "social disorders" might aid translational efforts to develop individualized clinical treatment approaches.

10.6 Closing Remarks

The study of epigenetics has raised much excitement in the field of behavioral neuroscience, as it provides a compelling mechanism underlying the interplay between psychosocial experience and molecular processes influencing gene expression. However, we have to remind ourselves that the field is still in its infancy (Mill and Heijmans 2013). We are just beginning to understand the complexities of an orchestrated symphony, which involves the integration of developmental and environmental signals to shape gene expression programs in a cell type specific manner. Epigenetic mechanisms involved in this orchestra include DNA methylation and modifications of the chromatin to make DNA regions more or less accessible to transcription. Another level of regulation involves micro RNAs (miRNAs). To what extent mRNA is translated into proteins is, among other mechanisms, under strict control of these short conserved noncoding RNAs with an average length of ~22 nucleotides. Their binding of mRNA sequences – predominantly in their 3′ untranslated region – exerts inhibition or repression on translational processes (Bartel 2004). In relation to the oxytocin receptor gene, it is of note that the mature 9 amino

acid nonapeptide hormone oxytocin stimulates miRNA expression in human myometrium (Cook et al. 2015), and multiple miRNA binding sites in the 3′UTR of the *OXTR* mRNA are predicted. Interestingly, it was shown that *OXT* mRNA is targeted by miR-24 in the mouse hypothalamus reducing oxytocin transcript and protein levels (Choi et al. 2013a). Furthermore, the 897 bp oxytocin gene (*OXT*; NM_000915.3- coding for the precursor protein oxytocin-neurophysin-I) itself is embedded into a CGI on chromosome 20. Thus, oxytocin release and signaling might also be dynamically influenced by epigenetic mechanisms.

Although all these gene regulatory mechanisms can be influenced by variation in the environment, and although epigenetics is thought of as the biological substrate of a gene–environment interaction, it is important to remember that these processes do not occur independently of the genomic context. For instance, genotype explains about 80 % of the variation in DNA methylation (Gertz et al. 2011), and single SNPs, so-called methylation quantitative trait loci (mQTLs; Zhang et al. 2014), can directly influence DNA methylation (Moser et al. 2008).

Another level of regulation is achieved via posttranslational modifications. On the protein level, the 389 amino acid human OXTR protein shows multiple posttranslational modification sites. Several phosphorylation sites are described (at threonine 235; serine 366/368/369/370) which lead to further protein activation. Glycosylation is one of the most common posttranslational modifications to occur in protein biosynthesis which leads to protein stabilization and correct folding and can occur at OXTR asparagine 8, 15, and 26, whereas ubiquitination, a common signal for protein degradation, is described to occur at OXTR lysine 234. Thus, different posttranslational protein modifications work together in order to dynamically activate or inactivate protein activity to influence oxytocin signaling processes.

All these above mentioned regulatory processes are constantly influenced by environmental factors, which have the capability to fine-tune gene regulatory mechanisms in interaction with the underlying genotype in a time and tissue-specific manner. Variability in DNA methylation levels represents just one of the involved mechanisms, and any oversimplification of gene regulatory mechanisms should be avoided. Better understanding of complete gene regulatory mechanisms ranging from variation in the linear DNA sequence to accessibility of DNA up to activated protein, together with the understanding of how disturbances in these processes are mechanistically linked to diseases processes, might represent one of the most challenging task of psychiatric genetics in the future.

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Chapter 11
Psychosocial Stress and DNA Methylation

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Abstract Psychosocial stress has profound effects on physical and mental health. Recent evidence suggests that this association can be epigenetically mediated. Exposure to psychosocial stress, particularly early in life, might trigger alterations in the epigenome, such as changes in DNA methylation. In this chapter we will summarize human epigenetic research assessing DNA methylation changes related to psychosocial stress exposure, with a focus on early life adversities. Various epigenetic studies investigated maternal psychosocial stress or mood disturbances during pregnancy in relation to the offspring's epigenome at birth or later in life or child maltreatment, adverse socioeconomic conditions, or stressful life events during childhood in relation to DNA methylation, in postmortem brain tissue, peripheral blood, saliva, and buccal epithelial cells, later in life. Although many of these studies indicate that alterations in DNA methylation persist from early life until adolescence or even adulthood, recent evidence also suggests that the human methylome might remain dynamically regulated by psychosocial experiences even beyond childhood. Interestingly, psychosocial stress across different age ranges was linked to changes in DNA methylation of genes implicated in the stress response system, such as the glucocorticoid receptor gene (*NR3C1*); FK506 binding protein gene (*FKBP5*); serotonin transporter gene (*SLC6A4*); genes involved in development, including the brain-derived neurotrophic factor gene (*BDNF*); parentally imprinted genes; and genes involved in the immune system. We here review selected findings from this rapidly growing research field and discuss limitations as well as potential implications for research and clinical practice.

Keywords Acute stress • Chronic stress • DNA methylation • Childhood adversities • Epigenetics • Prenatal adversities

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11.1 Psychosocial Stress and Relevance for Mental Health

Exposure to chronic or severe psychosocial stress, particularly during early development, is one of the major risk factors for developing a mental disorder (Kessler et al. 1997; Green and McLaughlin 2010; McLaughlin et al. 2010). Psychosocial stress exposure disrupts an organism's psychological and physiological balance, which needs to be reestablished through processes of stress adaptation (Selye 1950). These adaptation processes rely on the orchestrated regulation of psychological and physiological, including neuroendocrine, stress response systems (Chrousos and Gold 1992). A dissonance in the interplay of these stress response systems leads to an impaired stress adaptability, subsequently threatening mental health (Hellhammer and Hellhammer 2008). Recent epigenetic studies suggest that psychosocial stress might influence the epigenome with consequences for stress response systems and physical and mental health.

The detrimental effects of psychosocial stress exposure can be observed as early as during fetal development: intrauterine exposure to psychosocial stress of the mother during pregnancy negatively affects birth outcome, child behavior, and health later in life (Schlotz and Phillips 2009; Entringer et al. 2010; Tegethoff et al. 2010, 2011). During childhood, adverse psychosocial experiences – such as interpersonal loss, family difficulties, exposure to violence or neglect, and physical or economic adversity and disaster – might overwhelm a child's stress coping resources and induce a chronic state of stress (Green and McLaughlin 2010). But also during adulthood, traumatic experiences and chronic stress exposure increase the risk to develop mental disorders as well as physical diseases (Meinlschmidt et al. 2009).

11.2 Psychosocial Stress and Epigenetics

A decade ago, a series of animal studies on variations in the early rearing environment paved the way for a new area in psychobiological research. These animal studies reported epigenetic differences in stress-related genes in the hippocampus of rats exposed to an adverse versus supportive early rearing environment. Specifically, offspring of rat mothers exhibiting low levels of licking and grooming (LG) showed increased hippocampal DNA methylation of a regulatory CpG site located at an NGFI-A (nerve growth factor-induced protein A; a transcription factor) consensus region of the glucocorticoid receptor (*GR*) exon 1_7 promoter (Weaver et al. 2004). The hypermethylation and the associated histone modifications resulted in decreased NGFI-A binding to its consensus region (Weaver et al. 2004). These epigenetic modifications reduced *GR* expression causing decreased hypothalamic pituitary adrenal (HPA) axis feedback sensitivity. Consequently, offspring of low LG mothers showed an exaggerated stress response and augmented fear behavior in behavioral tests (Liu et al. 1997; Francis et al. 1999; Caldji et al. 2011). These results demonstrated that epigenetic modifications in the *GR* could mediate the association

of low maternal LG with augmented stress reactivity. Notably, the effects of maternal LG were sustained in cross-fostering experiments (Francis et al. 1999; Weaver et al. 2004; Cameron et al. 2008), which ruled out an exclusive role of epigenetic inheritance. Furthermore, subsequent experiments demonstrated that the effects induced by low maternal LG were reversed by pharmacological treatment targeting the closed chromatin structure, which resulted in epigenetic patterns, hippocampal *GR* expression, GR protein levels, and stress responses comparable to offspring raised by high LG mothers (Weaver et al. 2004).

These animal studies on epigenetic programming by the early psychosocial environment inspired subsequent research to examine psychosocial adversities and epigenetic processes. Today, animal and human studies indicate that a variety of psychosocial adversities are associated with epigenetic modifications in a broad range of genes related to the stress response, immune function, and other biological systems.

This chapter will summarize findings on epigenetic outcomes in children and adults who were exposed to psychosocial stress (i) in utero, (ii) during childhood, and (iii) in adulthood. We will focus on DNA methylation as one major epigenetic process and on findings from human studies. Finally, we will discuss potential implications for future research and clinical practice.

11.3 Prenatal Stress and DNA Methylation

Offspring of mothers who suffered from severe psychosocial stress or mental disorders during pregnancy have an increased risk for poor birth outcome (Engel et al. 2005; Tegethoff et al. 2010; Dancause et al. 2011; Bolten et al. 2011; Baibazarova et al. 2013). In turn, unfavorable birth parameters, as well as intrauterine exposure to increased maternal cortisol levels and mood disturbances, are related to diminished neuronal, cognitive, and behavioral outcomes of the child (Brand et al. 2006; Martini et al. 2010; Dancause et al. 2011; Tegethoff et al. 2011; Li et al. 2012; Meinlschmidt and Tegethoff 2015). Potential biological mechanisms contributing to this phenomenon are described within the framework of the "developmental origins of health and disease hypothesis". Originally based on the observation that low birth weight infants are at a greater risk for morbidity and premature mortality (Barker 2007) and the finding that malnutrition in utero increased the risk for a variety of diseases later in life (Barker 1998), this hypothesis states that early life adversities, including those occurring prenatally, increase the risk for later physical and mental disorders, presumably by inducing processes of adaptations in physiological, metabolical, and other biological systems (Tegethoff 2009). Related to this notion, the "predictive adaptive response model" argues that the intrauterine environment might induce processes of adaptations in the developing offspring based on cues from the maternal environment (Gluckman et al. 2005). These biological adaptations might partially involve epigenetic modifications affecting gene regulation and expression with consequences for infant stress adaptation and development,

supporting the concept of an "epigenetic programming" by environmental factors (Oberlander et al. 2008; Gluckman et al. 2011; Bromer et al. 2012). Indeed, epigenome-wide studies support the idea of an intrauterine epigenetic programming of the developing organism by the maternal environment (Teh et al. 2014). To date, research suggests that a range of maternal psychosocial adversities and maternal mental well-being during pregnancy affects the child's epigenome.

11.3.1 Maternal Psychosocial Adversities During Pregnancy

A developing organism might adapt to its potential future environment based on cues of the intrauterine environment (Gluckman et al. 2005). These adaptions may be beneficial, especially, if the prediction of the future environment is accurate. However, the adaptations might be unfavorable and associated with certain costs, for example, in cases where (i) the intrauterine environment does not match the future environment or where (ii) preparing for an untoward future environment prevents certain adverse outcomes but impedes normal growth and development. Hence, adjustment to a stressful maternal environment might be maladaptive for the future environment. Several studies suggest epigenetic adaptations in utero as a mechanism for the transmission of consequences of maternal psychosocial experiences from mother to child.

11.3.1.1 Intimate Partner Violence

Maternal experience of intimate partner violence during pregnancy, as reported 10–19 years later, predicted DNA methylation at the glucocorticoid receptor gene (*NR3C1*) promoter in blood samples of adolescent offspring (Radtke et al. 2011). Interestingly, intimate partner violence before and after pregnancy did not predict offspring *NR3C1* DNA methylation, indicating that programming occurred in utero rather than during early childhood. Of note, intimate partner violence before, during, and after pregnancy was not associated with maternal *NR3C1* methylation, and there was no correlation between maternal and child DNA methylation in this promoter region, indicating mechanisms beyond heritability. Remarkably, maternal stress during pregnancy predicted *NR3C1* methylation in adolescents, suggesting that these epigenetic modifications of an essential HPA axis regulating gene might persist into adulthood.

11.3.1.2 Stress Related to War and Political Conflicts

Prenatal exposure to war stress predicted DNA methylation of *NR3C1* exon 1_F – an area homologous to the rat *GR* exon 1_7 – in umbilical cord blood, as well as in maternal peripheral blood at birth (Mulligan et al. 2012). This study tested

three prenatal stressors related to maternal exposure to the Congolese war, including maternal deprivation (financial stress), mundane stress (daily stress), and war stress (war-related events, such as rape, refugee status, family member killed). Although all three stressors predicted cord blood *NR3C1* methylation, war stress had the strongest predictive value. Additionally, birth weight was associated with maternal stress experiences and cord blood *NR3C1* methylation, proposing DNA methylation of this region as a potential mediator in the association between maternal stress and newborn risk phenotype. Similar to the study by Radtke and colleagues (2011), maternal stress experience was not related to maternal DNA methylation profile. Additionally, DNA methylation profiles differed between mother and child, suggesting that DNA methylation at the assessed locus is susceptible to environmental conditions and not purely genetically determined.

Similarly, Perroud and colleagues (2014) compared DNA methylation of *NR3C1* and *NR3C2* (mineralocorticoid receptor gene) between mother-child dyads exposed to the Tutsi genocide during pregnancy and unexposed dyads. Psychological data and peripheral blood samples for DNA methylation and protein level analysis were collected when the children were adolescents. Exposure to genocide resulted in increased symptom severity for PTSD and depression at time of assessment. Furthermore, exposed mothers and children showed lower cortisol and glucocorticoid receptor levels and higher mineralocorticoid receptor levels. This indicates a potential psychological as well as biological transmission of the traumatic experiences. Correspondingly, exposed offspring, as well as their mothers, showed increased *NR3C1* methylation compared to unexposed dyads. In general and in line with the previous studies, exposed children showed the highest DNA methylation values at individual *NR3C1* CpG sites as compared to their exposed mothers or to unexposed mothers. *NR3C2* methylation was comparable between exposed and unexposed subjects. In contrast to some studies mentioned previously (Radtke et al. 2011; Mulligan et al. 2012), there was a correlation between maternal and offspring *NR3C1* methylation. This association was only significant in the unexposed group, although the exposed group might not have shown any correlation due to ceiling effects. Explanations for a potential association might be epigenetic inheritance from mother to child or epigenetic adaptations induced by a shared environment during the child's first 17–18 years of life.

11.3.1.3 Perceived Stress

Two recent studies examined prenatal stress and offspring epigenetic programming of genes beyond *NR3C1*: DNA methylation at imprinted regulatory regions (Vidal et al. 2014) – genes expressed from one parental allele only – and the serotonin transporter gene (*SLC6A4*) (Wankerl et al. 2014).

Vidal and colleagues (2014) investigated DNA methylation at eight differentially methylated regions of parentally imprinted genes, which are relevant for early growth and development, including insulin-like growth factor 2 (*IGF2*), maternally

expressed gene 3 (*MEG3*), mesoderm-specific transcript homolog protein (*MEST*), paternally expressed genes 3 and 10 (*PEG3*, *PEG10*), neuronatin (*NNAT*), non-protein coding gene *H19*, and pleiomorphic adenoma gene-like 1 (*PLAGL1*). The authors hypothesized that DNA methylation of the assessed regions would explain the association between prenatal stress and preterm birth. Although this hypothesis could not be confirmed, high levels of perceived stress during pregnancy predicted increased cord blood DNA methylation of a differentially methylated region associated with *MEST*, a gene implicated in maternal care, growth retardation, and obesity. Similarly, maternal stress during pregnancy predicted greater DNA methylation in 4 out of 83 CpG sites located within a CpG island in *SLC6A4* in peripheral blood samples of healthy young adults (Wankerl et al. 2014).

11.3.1.4 Natural Disaster

Offspring born to mothers who were pregnant during the Quebec Ice Storm in 1989, one of the worst natural disasters in Canadian history, showed changes in T-cell DNA methylation profiles compared to offspring of unexposed mothers (Cao-Lei et al. 2014). Specifically, degree of maternal objective hardship during pregnancy, but not subjectively rated levels of stress, was related to epigenetic profiles in 13-year-old offspring. The hardship-associated modifications in DNA methylation were observed in 1675 CpG sites associated with 957 genes. A high proportion of the discovered genes were implicated in immune processes. Remarkably, the objectively measured hardship associated with the disaster (e.g. different regions were unequally affected) imitates randomly assigned hardship conditions and therefore simulates a certain degree of experimental control. Interestingly, this study demonstrated that hardship condition, rather than subjective levels of stress, predicted DNA methylation profiles.

In sum, studies on prenatal psychosocial stress exposure support the notion of a fetal epigenetic programming hypothesis. Prenatal stress might be transmitted to the developing fetus triggering epigenetic adaptations. However, the mechanisms of transmission still need to be elucidated. For example, maternal cortisol is inactivated in the placenta to reduce cortisol exposure of the offspring in utero. This might be epigenetically mediated as well: socioeconomic adversity during pregnancy was related to decreased DNA methylation of the gene encoding the enzyme responsible for maternal cortisol inactivation in the placenta, 11-beta hydroxysteroid dehydrogenase enzyme type 2 (*HSD11B2*) (Appleton et al. 2013). This might protect the developing organism from exposure to excess levels of maternal cortisol. Nevertheless, the aforementioned studies suggest that signals of a potentially stressful environment are transmitted to the offspring in utero with consequences for epigenetic patterns of *NR3C1* and genes involved in growth and development and the immune system. Therefore, the nature and translation of stress signals from the mother to the child's epigenome should be addressed in future studies. A summary of the discussed studies is illustrated in Fig. 11.1 and Table 11.1.

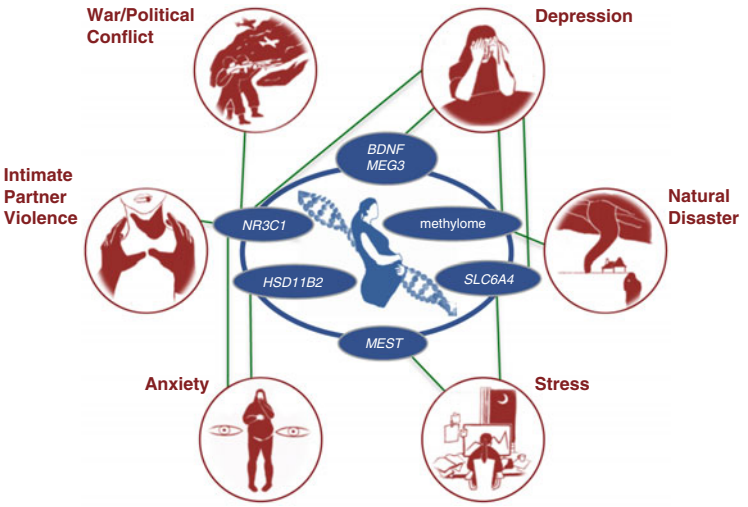

Fig. 11.1 Prenatal maternal stress exposure is associated with offspring DNA methylation of several candidate genes and on a methylome-wide level at birth and later in life. Maternal factors include psychosocial stress, such as exposure to intimate partner violence, war and political conflicts, natural disasters and stress, as well as maternal mood, including maternal depression and anxiety. The genes, which might be modified by prenatal maternal adversities are indicated in the *blue circles*. Gene abbreviations: *BDNF* brain-derived neurotrophic factor, *HSD11B2* 11-beta hydroxysteroid dehydrogenase enzyme type 2, *MEG3* maternally expressed gene 3, *MEST* mesoderm-specific transcript, *NR3C1* glucocorticoid receptor, *SLC6A4* serotonin transporter. Illustrations by Christoph Unternaehrer

11.3.2 Maternal Mood During Pregnancy

Maternal mood disturbances during pregnancy, such as depression or anxiety, are associated with a broad range of unfavorable child outcomes. Similar to exposure to maternal stress in utero, low maternal mood has been associated with disadvantageous maternal rearing style (Lovejoy et al. 2000) and might signal a potentially adverse early environment. There is evidence for maternal mood-related epigenetic adaptation in several tissues collected from the offspring at birth or during childhood and adolescence.

11.3.2.1 Maternal Depression

Maternal depressed mood during the second and third trimester of pregnancy was associated with higher *NR3C1* exon 1_F methylation in cord blood at birth, independent of exposure to antidepressant medication (Oberlander et al. 2008). Notably, maternal depression during the third trimester was related to higher cord blood DNA methylation at a CpG located at the NGFI-A binding site. Moreover, increased DNA methylation at this particular site predicted increased infant HPA axis reactivity

Table 11.1 Summary of research findings on psychosocial stress and DNA methylation in humans

Psychosocial stressor(s)	Examined gene(s)/ genomic regions	Main finding (stress exposed versus unexposed)	Assessed tissue, developmental timing of sample collection (and information on study subjects)	Reference
Prenatal adversity				
Maternal intimate partner violence	*NR3C1*	Hypermethylation	Peripheral blood of adolescents	Radtke et al. (2011)
Maternal exposure to war-related stress	*NR3C1*	Hypermethylation	Cord blood at birth	Mulligan et al. (2012)
Maternal exposure to genocide-related stress	*NR3C1, NR3C2*	*NR3C1* hypermethylation	Peripheral blood of adolescents	Perroud et al. (2014)
Maternal perceived stress	*IGF2, MEG3, MEST, PEG3, PEG10, NNAT, H19, PLAGL1*	*MEST* hypermethylation	Cord blood at birth	Vidal et al. (2014)
Maternal perceived stress	*SLC6A4*	Hypermethylation	Peripheral blood of young adults	Wankerl et al. (2014)
Maternal hardship associated with natural disaster	Methylome	Modifications in 957 genes (1675 CpGs), genes mostly related to immune processes	Saliva of teenagers	Cao-Lei et al. (2014)
Maternal socioeconomic adversity	*HSD11B2*	Hypomethylation	Placenta at birth	Appleton et al. (2013)
Maternal depression and anxiety	*NR3C1*	Hypermethylation	Cord blood at birth	Oberlander et al. (2008)
Maternal depression	*NR3C1*	Hypermethylation	Placenta at birth	Conradt et al. (2013)
Maternal depression	*NR3C1, BDNF*	*NR3C1* hypermethylation in males, *BDNF* hypomethylation in males and females	Buccal epithelial cells at 2 months after birth	Braithwaite et al. (2015)
Maternal depression	*SLC6A4, BDNF*	*SLC6A4* hypermethylation	Cord blood at birth	Devlin et al. (2010)
Maternal depression	*MEG3, MEST, IGF2, PEG3, PEG10, H19, NNAT, PLAGL1*	*MEG3* hypermethylation	Cord blood at birth	Liu et al. (2012)

Maternal lifetime history of depression	Methylome	Modifications at 145 CpGs (FDR < 0.05), 2520 CpGs (FDR < 0.1), CpGs implicated in stress and immune system	Cord blood at birth	Nemoda et al. (2015)
Maternal anxiety and cortisol levels	*NR3C1*	Mostly hypermethylation	Cord blood at birth	Hompes et al. (2013)
Maternal anxiety	*HSD11B2*	Hypermethylation	Placenta at birth	Conradt et al. (2013)
Childhood adversity in form of maltreatment and violence				
Maltreatment	*rRNA*	Hypermethylation	Hippocampus of adult suicide victims and controls	McGowan et al. (2008)
Maltreatment	*NR3C1*	Hypermethylation	Hippocampus of adult suicide victims and controls	McGowan et al. (2009)
Maltreatment	*NR3C1*	Hypermethylation exons 1B and 1C, hypomethylation exon 1H	Hippocampus of adult suicide victims and controls	Labonté et al. (2012)
Maltreatment	6.5 Mb centered around *NR3C1*	Bidirectional modifications	Hippocampus of adult suicide victims and controls	Suderman et al. (2012)
Maltreatment	*NR3C1*	Hypermethylation	Peripheral blood of adults with different psychopathological conditions	Perroud et al. (2011)
Maltreatment	*NR3C1*	Hypermethylation	Peripheral blood of adults	Tyrka et al. (2012)
Physical and sexual violence	*NR3C1*	No modifications	Peripheral blood of adult women suffering from bulimia nervosa (and borderline personality disorder) and healthy controls	Steiger et al. (2013)
Physical and sexual violence	*FKBP5*	Hypomethylation	Peripheral blood of adult *FKBP5* risk allele carriers	Klengel et al. (2013)

(continued)

Table 11.1 (continued)

Psychosocial stressor(s)	Examined gene(s)/ genomic regions	Main finding (stress exposed versus unexposed)	Assessed tissue, developmental timing of sample collection (and information on study subjects)	Reference
Physical and sexual violence	*SLC6A4*	Hypermethylation	Lymphoblast cell lines from adults	Beach et al. (2010)
Sexual violence	*SLC6A4*	*SLC6A4* methylation mediates association between sexual violence during childhood and later antisocial personality disorder	Lymphoblast cell lines from adults	Beach et al. (2011)
Sexual violence	*SLC6A4*	Hypermethylation	Lymphoblast cell lines from adults	Vijayendran et al. (2012)
Sexual violence	*SLC6A4*	Hypermethylation	Lymphoblast cell lines from adult women	Beach et al. (2013)
Physical or sexual violence	*SLC6A4*	Hypermethylation	Peripheral blood of adults	Kang et al. (2013)
Maltreatment	*SLC6A4*	No modifications	Peripheral blood of adults	Wankerl et al. (2014)
Maltreatment	*BDNF*	Hypermethylation	Peripheral blood of adults	Perroud et al. (2013)
Sexual violence	*DRD2*	Hypermethylation	Peripheral blood of adult women with and without bulimia-spectrum disorder	Groleau et al. (2014)
Maltreatment	Methylome	Modifications at 2868 CpGs, genes implicated in neoplasm and various biological pathways	Saliva of adults	Yang et al. (2013)
Adversities	Methylome	Modifications in distinct pathways depending on presence of history of childhood adversities	Peripheral blood of PTSD patients and healthy controls	Mehta et al. (2013)

Maltreatment	Methylome	Bidirectional modifications	Peripheral blood of adults suffering from borderline personality disorder or depression	Prados et al. (2015)
Childhood adversity related to family environment				
Poor parental care, parental loss	*NR3C1*	Hypermethylation	Peripheral blood of adults	Tyrka et al. (2012)
Low maternal warmth and affection, rejection	*NR3C1, MIF*	Hypermethylation	CD3 T cells of young adults	Bick et al. (2012)
Low maternal care	*BDNF, OXTR*	Hypermethylation	Peripheral blood of adults	Unternaehrer et al. (2015)
Parental loss	*NR3C1*	Hypermethylation	Saliva of adult women carrying a *MAOA* risk allele	Melas et al. (2013)
Parental loss	*SLC6A4*	Hypermethylation	Peripheral blood of adult depressed subjects	Kang et al. (2013)
Foster care placement	Methylome	Modifications at 180 CpGs of 173 genes, many genes involved in immune function	CD3 T cells of young adults	Bick et al. (2012)
Institutionalization	Methylome	Modification at 914 CpGs of genes involved in immune function and cellular signaling	Peripheral blood of children	Naumova et al. (2012)
Parental stress	Methylome	Modifications at 1–139 CpGs depending on timing of parental stress and parent (maternal versus paternal stress)	Buccal epithelial cells of adolescents	Essex et al. (2013)
Adverse childhood socioeconomic status and peer environment				

(continued)

Table 11.1 (continued)

Psychosocial stressor(s)	Examined gene(s)/ genomic regions	Main finding (stress exposed versus unexposed)	Assessed tissue, developmental timing of sample collection (and information on study subjects)	Reference
Low socioeconomic status	Methylome	Modifications at three CpGs	Peripheral blood of adults	Lam et al. (2012)
Very low socioeconomic status	Methylome	Modifications at 1252 gene promoter regions, genes involved in MAPK and chemokine pathway	Peripheral blood of adult men	Borghol et al. (2012)
Low family income and single parent household	*SAT2*, Alu element	Hypermethylation	Peripheral blood granulocytes of adult women	Tehranifar et al. (2013)
Cumulative socioeconomic risk	*SLC6A4*	Modifications at *SLC6A4* (effects partially depending on *SLC6A4* genotype and gender)	Lymphoblast cell lines from adolescent African Americans	Beach et al. (2014)
Being bullied	*SLC6A4*	Hypermethylation	Buccal epithelial cells in childhood	Ouellet-Morin et al. (2013)
Chronic and lifetime stress during adulthood				
Perceived stress	*COMT, LINE-1*	Hypomethylation at *COMT* SNP but no change at *COMT* promoter region or *LINE-1*	Peripheral blood of adult *COMT* Val/Val carriers	Ursini et al. (2011)
Work stress	*SLC6A4*	Hypomethylation	Peripheral blood of adult nurses	Alasaari et al. (2012)
Recent life stress	*SLC6A4*	No changes	Peripheral blood of adults	Wankerl et al. (2014)
Low socioeconomic status and manual workers	Global methylation	Hypomethylation	Peripheral blood of adults	McGuinness et al. (2012)
Low socioeconomic status	Methylome	Modifications at 545 gene promoter regions	Peripheral blood of adult men	Borghol et al. (2012)

Perceived stress	*NR3C1*	Hypermethylation	Peripheral blood of adults	Tyrka et al. (2012)
Perceived stress	*SLC6A4*	Hypermethylation	Peripheral blood of depressed adults	Kang et al. (2013)
Perceived stress and salivary cortisol	Methylome	Modifications in DNA methylation patterns	Peripheral blood of adults	Lam et al. (2012)
Acute psychosocial stress during adulthood				
Trier Social Stress Test	*BDNF, OXTR*	Dynamic modifications in *OXTR* after stress exposure	Peripheral blood of adults	Unternaehrer et al. (2012)
Trier Social Stress Test	*PRF1*	Hypermethylation after stress exposure	Peripheral blood of adults with and without chronic fatigue syndrome	Falkenberg et al. (2013)

Abbreviations: *BDNF* brain-derived neurotrophic factor, *COMT* catechol-O-methyltransferase, *CpG* cytosine-guanine dinucleotide, *DRD2* dopamine receptor D2, *FDR* false discovery rate, *FKBP5* FK506 binding protein 5, *H19* non-protein coding gene H19, *HSD11B2* 11-beta hydroxysteroid dehydrogenase enzyme type 2, *IGF2* insulin-like growth factor 2, *LINE-1* long interspersed nuclear element 1, *MAOA* monoamine oxidase A, *MEG3* maternally expressed gene 3, *MEST* mesoderm-specific transcript homolog protein, *MIF* macrophage migration inhibitory factor, *NNAT* neuronatin, *NR3C1* glucocorticoid receptor, *OXTR* oxytocin receptor, *PEG3* paternally expressed gene 3, *PEG10* paternally expressed gene 10, *PLAGL1* pleiomorphic adenoma gene-like 1, *PRF1* perforin 1, *PTSD* post-traumatic stress disorder, *rRNA* ribosomal RNA, *SAT2* spermidine/spermine N1-acetyltransferase family member 2, *SLC6A4* serotonin transporter

to stress at 3 months of age. Maternal depression was not associated with maternal *NR3C1* methylation status, which was also uncorrelated with cord blood DNA methylation status. Some of these findings on depression and *NR3C1* epigenetic modifications were replicated in a subsequent study reporting increased DNA methylation within the same *NR3C1* exon 1_F region in the placenta of mothers who were depressed during pregnancy (Conradt et al. 2013). Moreover, greater *NR3C1* methylation predicted decreased self-regulation competence in infants born to depressed mothers, as well as hypotonia and lethargy in infants from depressed and nondepressed mothers. Results from these two studies on *NR3C1* methylation suggest that maternal depression affects the offspring's epigenome with consequences for offspring stress reactivity and behavior. Lastly, prenatal depressive symptoms predicted greater *NR3C1* exon 1_F methylation in male infants as well as lower brain-derived neurotrophic factor (*BDNF*) exon IV methylation in male and female infants as measured in buccal epithelial cells at two months after birth (Braithwaite et al. 2015).

Besides *NR3C1*, research on maternal depression during pregnancy and DNA methylation has covered a few additional candidate genes, implicated in the serotonergic system, neurotrophic functions, and imprinted differentially methylated regions (DMRs). In more detail, increased maternal depression during the second trimester predicted decreased DNA methylation in the *SLC6A4* promoter region but not in the *BDNF* promoter in leukocytes collected from infant umbilical cord blood and maternal peripheral blood (Devlin et al. 2010). Similarly, severe maternal depression during pregnancy predicted higher DNA methylation at *MEG3* in infant cord blood but not at other examined DMRs, such as *IGF2*, *PEG3*, *PEG10*, *H19*, *NNAT*, and *PLAGL1* (Liu et al. 2012). Additionally, low birth weight infants had lower DNA methylation of *IGF2*, while high birth weight infants showed a higher DNA methylation of *PLAGL1* and *PEG10* compared to normal birth weight infants. However, DNA methylation within the assessed regions did neither mediate nor influence the association between maternal depressed mood during pregnancy and offspring weight at birth as hypothesized by the authors.

Finally, an epigenome-wide study identified 145 CpG sites at FDR $p<0.05$ (false discovery rate) or 2520 CpG sites at FDR $p<0.1$, respectively, that were differentially methylated in cord blood samples of babies born to mothers with a lifetime history of depression compared to never depressed mothers (Nemoda et al. 2015). The identified CpG sites were primarily located in genes involved in immune function and stress regulation, including *NR3C1*. Additionally, the genes were functionally overlapping with differentially methylated regions identified in postmortem hippocampal samples of adult males born to mothers with a history of depression compared to those without maternal depression history.

In short, signals of maternal depressive symptoms during pregnancy seem to be transmitted to the child in utero: prenatal exposure to maternal depression was associated with changes in DNA methylation of several CpG sites located within several stress-relevant genes, such as *NR3C1*, *SLC6A4*, *BDNF*, *MEG3*, and genes involved in immune functioning.

11.3.2.2 Maternal Anxiety

Maternal anxiety during pregnancy is another potential factor programming the child's epigenome (Oberlander et al. 2008). Indeed, levels of maternal anxiety during the third trimester predicted *NR3C1* methylation at a CpG site neighboring the NGFI-A binding site (Oberlander et al. 2008). These results were extended to different indicators of maternal anxiety and cortisol levels during all pregnancy trimesters acting as predictors of DNA methylation status of *NR3C1* exons 1_B, 1_D, and 1_F in cord blood at birth, whereby pregnancy-related anxiety was the best predictor for DNA methylation across the examined CpG sites (Hompes et al. 2013). Specifically, pregnancy-related anxiety at each trimester predicted (mostly increased) cord blood DNA methylation at several CpG sites within *NR3C1* exons 1_D and 1_F. However, this association between maternal anxiety during pregnancy and *NR3C1* methylation could not be confirmed in placental tissue (Conradt et al. 2013). Yet maternal anxiety was linked to increased DNA methylation in a CpG site of *HSD11B2* (Conradt et al. 2013). Moreover, infants of mothers suffering from anxiety during pregnancy and with low DNA methylation at *HSD11B2* in the placenta showed less hypotonia as compared to infants with high DNA methylation at this CpG site or born to mothers without anxiety during pregnancy.

Taken together, epigenetic research on maternal mood during pregnancy, especially during the second and third trimester, suggests mood-related alterations in DNA methylation of genes involved in HPA axis regulation, the serotonergic system, neurotrophins, immune function, and in the regulation of imprinted regions, with a few partially inconsistent results. Some of these epigenetic modifications were shown to be linked to infant cortisol response and self-regulation competence and might therefore be relevant for offspring development and health later in life. A summary of the discussed studies is illustrated in Fig. 11.1 and Table 11.1.

11.4 Psychosocial Stress During Childhood

Children experiencing severe or prolonged phases of psychosocial stress are at a higher risk for developing mental health problems across the lifespan (Lupien et al. 2009; Scott 2012; Carr et al. 2013). This association is illustrated by analyses based on the National Comorbidity Survey Replication (NCS-R), indicating that early adverse experiences accounted for 44.6 % of childhood-onset disorders and for around 30 % of later-onset mental disorders (Green and McLaughlin 2010), an association supported by further studies (Kessler et al. 2010). For example, a history of exposure to violence or neglect during childhood is a major predictor for the development of mental disorders later in life (Kessler et al. 2010). However, other features in the child's environment – for example, parental behavior, socioeconomic background, interpersonal loss, or family difficulties – also substantially contribute to the risk for mental health problems (Green and McLaughlin 2010).

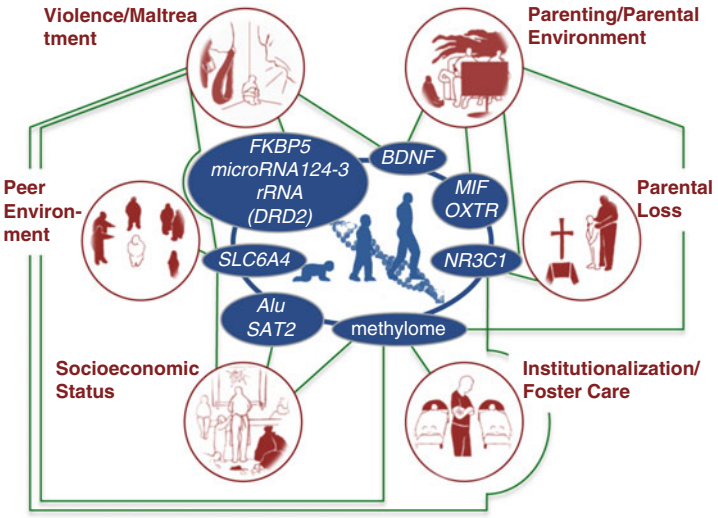

Fig. 11.2 Childhood stress exposure is associated with DNA methylation of several candidate genes and on a methylome-wide level. Investigated psychosocial stressors include exposure to violence and childhood maltreatment, parenting environment, socioeconomic status, parental loss, institutionalization, and peer environment. The genes, which might be modified by childhood adversities, are indicated in the *blue circles*. Gene abbreviations: *BDNF* brain-derived neurotrophic factor, *DRD2* dopamine receptor D2, *FKBP5* FK506 binding protein 5, *MIF* macrophage migration inhibitory factor, *NR3C1* glucocorticoid receptor, *OXTR* oxytocin receptor, *rRNA* ribosomal RNA, *SAT2* spermidine/spermine N1-acetyltransferase family member 2, *SLC6A4* serotonin transporter. Illustrations by Christoph Unternaehrer

The relationship between early psychosocial stress and the risk for mental disorders is partially mediated by developmental (mal)adaptations of psychobiological systems, particularly of those involved in the stress response (Tarullo and Gunnar 2006; Lupien et al. 2009; Andrews et al. 2013). Epigenetic programming of genes involved in these stress systems is a promising candidate mechanism to understand the molecular, biological, and behavioral consequences of psychosocial stress during childhood. Several studies examined childhood psychosocial stress and epigenetic patterns in stress-related candidate genes or across the epigenome in a range of target tissues, including brain, blood, saliva, and buccal epithelial cells. The presented findings are summarized in Table 11.1 and Fig. 11.2.

11.4.1 Maltreatment During Childhood

Child maltreatment, such as physical, sexual, and emotional violence, as well as physical and emotional neglect, is a major risk factor to develop mental disorders. Recent epigenetic studies suggest that early maltreatment is linked to adaptations in DNA methylation profiles of stress-related genes across different tissues.

11.4.1.1 Maltreatment During Childhood: Human Postmortem Studies

The first human postmortem study published on epigenetic adaptations related to adverse childhood experiences was conducted on tissue of adult suicide victims (McGowan et al. 2008). DNA methylation in the promoter region of a gene encoding for ribosomal RNA (rRNA) – a key player in protein synthesis in the cell – was increased in hippocampal tissue in suicide subjects with a childhood history of sexual or physical violence or severe neglect as compared to non-suicidal subjects without childhood maltreatment (controls). This finding was complemented by decreased rRNA expression in brain samples from suicide victims with a history of violence or neglect compared to non-suicidal and unexposed controls. A diagnosis of a mood disorder or substance abuse was not associated with differences in rRNA gene methylation, indicating that these effects might be attributed to early experiences rather than psychopathology. These findings were supplemented by a subsequent study that translated the animal findings of epigenetic programming of the hippocampal GR gene by maternal behavior (Weaver et al. 2004) to humans. Adult suicide victims with a history of childhood violence had increased DNA methylation at the *NR3C1* exon 1_F promoter region in hippocampal cells, as compared to suicide victims without a history of childhood violence and non-suicidal, unexposed controls (McGowan et al. 2009); this was accompanied by reduced total NR3C1 and exon 1_F specific expression levels in suicide victims with childhood exposure to violence, probably mediated by reduced NGFI-A binding to the examined *NR3C1* exon 1_F binding site. This effect was not explained by psychopathology or suicide per se, since DNA methylation status was comparable between controls and suicide victims without a history of childhood violence. The findings on aberrant epigenetic profiles in suicide victims exposed to childhood violence versus unexposed suicide victims and controls were extended from hippocampal *NR3C1* exon 1_F to other *NR3C1* exon 1 regions (Labonté et al. 2012). Distinct CpG sites located in the region of exons 1_B and 1_C were hypermethylated in suicide victims exposed to childhood violence, while sites within 1_H were hypomethylated as compared to unexposed suicide victims or controls. Likewise, increased DNA methylation in *NR3C1* exons 1_B and 1_C was associated with decreased total and variant specific expression levels, whereas increased *NR3C1* exon 1_H methylation was related to increased total and variant specific expression, demonstrating different consequences of DNA methylation in different regions of *NR3C1* exon 1. Moreover, experiencing violence during childhood might program an even wider region around *NR3C1*, which might exhibit a comparable epigenetic sensitivity across different species (Suderman et al. 2012). In a subsequent study, the same authors investigated DNA methylation in multiple promoter areas across the whole genome in the hippocampus of suicide victims and non-suicidal controls (Labonté et al. 2013). They found that promoter regions of genes involved in cellular and neuronal plasticity were both hypermethylated and hypomethylated in the hippocampus of suicide victims compared to non-suicidal controls.

11.4.1.2 Maltreatment During Childhood: Human Peripheral Tissue

Assessing epigenetic profiles in peripheral tissue samples that can be collected in living humans offers a great potential to study epigenetic programming in subjects with a wide range of mental disorders and physical diseases. Also, epigenetic patterns of some genes might be correlated across tissues. For instance, results on epigenetic changes in *NR3C1* exon 1_F following childhood maltreatment seem to be similar for hippocampal tissue and peripheral blood specimen (Perroud et al. 2011).

HPA Axis Functioning Related Genes Childhood sexual violence was related to *NR3C1* exon 1_F hypermethylation in white blood cells in adults with various psychopathological backgrounds (Perroud et al. 2011). The association was dose dependent, with more severely and repeatedly maltreated individuals showing greater *NR3C1* methylation. In addition, adversities in form of physical violence and emotional neglect, as well as number of different types of childhood maltreatments, predicted increased *NR3C1* exon 1_F methylation status. An independent study replicated these findings, indicating that DNA methylation of the *NR3C1* promoter region 1_F was hypermethylated in blood leukocytes of adults reporting childhood maltreatment (Tyrka et al. 2012). In contrast, a study including a sample of women diagnosed with bulimia with or without comorbid borderline personality disorder and healthy women did not find any differences in *NR3C1* methylation in DNA extracted from peripheral whole blood that were related to exposure to childhood physical or sexual violence (Steiger et al. 2013).

FK506 binding protein 5 (*FKBP5*) is a gene regulating glucocorticoid receptor activity and function with consequences for HPA reactivity. A risk allele variant in a single nucleotide polymorphism (SNP) located in close proximity of a functional *FKBP5* glucocorticoid receptor response element (GRE) causes a structural change of the *FKBP5* gene, resulting in increased *FKBP5* expression and changes in glucocorticoid sensitivity (Binder et al. 2008). Carriers of the risk allele with childhood experiences of sexual or physical violence had a higher risk of lifetime posttraumatic stress disorder (PTSD) diagnosis and increased current PTSD symptom severity as compared to carriers of the protective allele (Klengel et al. 2013). Remarkably, child physical and sexual violence was associated with decreased DNA methylation at a region overlapping with a consensus GRE in intron 7 of *FKBP5* in peripheral blood of risk allele carriers compared to carriers of the protective allele. These results were partially validated in a second female-only cohort, with physical and emotional violence predicting decreased peripheral blood *FKBP5* methylation status within the same region in risk allele carriers compared to unchanged or increased DNA methylation in carriers of the protective allele (Klengel et al. 2013). Additional analyses suggested that the observed changes in DNA methylation of risk allele carriers with prior experience of childhood violence were (i) specific for childhood trauma compared to adulthood trauma exposure, (ii) stable over time, (iii) not mediated by trauma-related differences in cell-type composition, (iv) generalizable to hippocampal tissue, (v) linked to glucocorticoid resistance, (vi) related to global changes in gene expression of peripheral immune cells,

and (vii) correlated with morphological changes in brain structures relevant for stress reactivity.

Genes of the Serotonergic System *SLC6A4* is another gene that seems sensitive to exposure to violence during childhood: physical violence by parents or sexual violence by a family member predicted DNA methylation of a CpG island upstream from *SLC6A4* in lymphoblast cell lines from adult individuals (Beach et al. 2010). A subsequent study by the same group revealed lymphoblast *SLC6A4* methylation as a mediator between sexual violence by a family member and later antisocial behavior among women (Beach et al. 2011). Moreover, sexual violence during childhood predicted DNA methylation in several CpG sites in the *SLC6A4* promoter region as measured in lymphocytes from adults, with consequences for *SLC6A4* expression (Vijayendran et al. 2012). In fact, increased DNA methylation within the examined CpG island had previously been linked to decreased serotonin receptor expression levels in a *SLC6A4* genotype-dependent manner (Philibert et al. 2007). Finally, sexual violence during childhood and psychopathology of the biological parent interacted to predict DNA methylation at the same *SLC6A4* region in lymphocytes in adult women who were adopted during childhood (Beach et al. 2013). These epigenetic changes were associated with antisocial personality disorder, thus partially mediating the association between sexual violence during childhood and adult antisocial personality disorder. Of note, participants of all four studies examining *SLC6A4* methylation were recruited from the Iowa Adoption Studies and were therefore not biologically related to their social parents, hence excluding purely inheritable effects being responsible for the observed associations. The results on *SLC6A4* methylation have been validated in an independent sample examining depressed patients: subjects who reported childhood physical or sexual violence had greater *SLC6A4* promoter methylation in blood leukocytes (Kang et al. 2013). Furthermore, increased *SLC6A4* methylation was associated with higher perceived stress and depression severity. However, a more recent study could not find an association between early life psychosocial stress and *SLC6A4* CpG island methylation status (Wankerl et al. 2014), despite childhood maltreatment predicting *SLC6A4* expression levels.

Other Candidate Genes In a study comparing DNA methylation of the brain-derived neurotrophic factor gene (*BDNF*) between individuals suffering from borderline personality disorder and healthy controls, Perroud and colleagues (2013) found a dosage effect of number of childhood maltreatments on mean DNA methylation of *BDNF* exons I and IV in peripheral blood. A higher number of childhood violence and neglect predicted increased *BDNF* methylation. Additionally, subjects with borderline personality disorder had higher *BDNF* methylation levels in exons I and IV. Of note, a positive treatment response to dialectical behavior therapy was associated with decreased *BDNF* methylation status, whereas nonresponders had increased *BDNF* methylation levels after treatment.

Similarly, women with a history of sexual violence during childhood and suffering from a bulimia-spectrum disorder had greater DNA methylation of the dopamine

D2 receptor (*DRD2*) in peripheral blood DNA as compared to controls without any eating disorder and no experience of violence during childhood (Groleau et al. 2014). However, the difference between subjects exposed to sexual violence and those not exposed did not reach statistical significance, which – in light of the higher *DRD2* methylation in subjects with bulimia-spectrum and comorbid borderline personality disorder versus bulimia-spectrum without borderline personality disorder and healthy controls – suggests that the observed differences in DNA methylation might be, at least in part, attributed to the presence of the eating disorder rather than early adversities.

Epigenome-Wide Association Studies Studies examining DNA methylation across the entire genome promise to promote our understanding of epigenetic programming by early life adversities. The provision of new platforms for epigenetic analyzes enables researchers to investigate epigenetic patterns across thousands of CpG sites distributed across the human methylome. For example, in salivary cells, DNA methylation differed between maltreated children and non-maltreated children at 2868 CpG sites (after controlling for multiple testing) (Yang et al. 2013). Differentially methylated genes could be attributed to disease biomarkers for neoplasms and a broad variety of biological pathways. Furthermore, Mehta and colleagues (2013) compared epigenome-wide DNA methylation and gene expression profiles in peripheral blood of patients suffering from PTSD with a history of childhood adversities to subjects with PTSD without childhood adversities and to childhood adversity-exposed controls without PTSD. The differential expression profiles of PTSD cases with childhood adversities versus controls were almost non-overlapping with the differential expression profiles of PTSD cases with childhood adversities versus PTSD cases without childhood adversities. Transcriptional enrichment analysis indicated that distinct biological pathways were affected in the two PTSD groups. Moreover, in subjects with PTSD and childhood maltreatment, transcriptional differences were more strongly associated with epigenetic profiles compared to subjects with PTSD without childhood adversities. These findings suggest that trauma-related epigenetic changes during early development involve different biological pathways in comparison to adult trauma exposure, which might indicate distinct pathobiological processes associated with PTSD following childhood adversities.

Finally, severity of childhood maltreatment was associated with DNA methylation in multiple genomic regions assessed in blood leukocytes of subjects suffering from borderline personality disorder with high levels of childhood adversity and of subjects with major depressive disorder and low levels of childhood adversity (Prados et al. 2015). The most relevant result included a CpG site nearby microRNA 124-3 – a gene coding for a microRNA that targets multiple genes, including *NR3C1* – which was associated with severity of child maltreatment and borderline personality disorder.

In conclusion, child maltreatment, especially sexual violence during childhood, predicts DNA methylation of several genes in brain, blood, saliva, and buccal epi-

thelial cells, including *NR3C1*, *SLC6A4,* and a few other genes as identified using an epigenome-wide approach. The greater proportion of these studies suggests increased DNA methylation of genes involved in mental health and disease, indicating potentially (but not necessarily) reduced expression of these genes. These epigenetic changes might point to epigenetic adaptations related to early life adversities, which might be associated with modifications in biological stress adaptation processes and a risk for mental disorders in adulthood. However, future research has to address the exact molecular mechanisms whereby childhood adversities might program the epigenome and some of the results need replication.

11.4.2 Parenting Environment

The experience of parental warmth and care is vital for the healthy development of a child (Bowlby 1969). Epigenetic mechanisms might be involved in the biological pathways linking an adverse parental rearing environment to developmental processes, mental disorders, and physical diseases later in life. First evidence for an epigenetic programming by the early parental rearing environment originated from rat studies of maternal care (Weaver et al. 2004). Recent human studies support the assumption that these epigenetic findings from the rodent model might be translated to humans.

Parental Care Experience of poor parental care was linked to increased DNA methylation at the *NR3C1* promoter in blood leukocytes collected from adults (Tyrka et al. 2012). Remarkably, DNA methylation at this region was also linked to an attenuated cortisol response in a dexamethasone/corticotropin-releasing hormone (CRH) stress test. Similarly, maternal self-report of greater warmth and affection toward their child were correlated with decreased offspring *NR3C1* and *MIF* (macrophage migration inhibitory factor, which is implicated in glucocorticoid signaling and inflammatory processes) methylation in CD3 T cells collected up to 10 years later (Bick et al. 2012). Greater DNA methylation of the investigated CpG site in *MIF* was also related to children's reports of maternal rejection. Furthermore, low maternal care during childhood and adolescence was associated with increased DNA methylation measured in a target sequence in *BDNF* exon VI and in oxytocin receptor (*OXTR*) exon III in peripheral blood samples of adults, independent of blood cell-type composition (Unternaehrer et al. 2015). These results suggest epigenetic programming of two genes relevant for neuronal plasticity and protection and a hormone receptor involved in the regulation of the stress system.

Parental Loss Likewise to low parental care, parental loss predicted increased DNA methylation at the *NR3C1* promoter region (Tyrka et al. 2012). These findings were confirmed in an independent study (Melas et al. 2013), which additionally took genotype of monoamine oxidase A (*MAOA*) into account, a gene encoding for a mitochondrial enzyme metabolizing neurotransmitters, such as dopamine and

serotonin. Women carrying a risk allele in a functional length polymorphism of *MAOA* and who experienced death of a parent during childhood showed greater *NR3C1* exon 1_F promoter methylation measured in salivary cells, as compared to women carrying the protective allele or exposed to other early adversities, including parental divorce, financial problems, or other familial constraints. Finally, parental loss during childhood predicted increased *SLC6A4* promoter methylation in depressed patients (Kang et al. 2013).

Parental Separation Individuals with foster care placement during childhood showed differential DNA methylation at 180 CpG sites located in 173 genes across the genome, as compared to young adults who were not placed into foster care (Bick et al. 2012). Many of the identified genes were involved in metabolic pathways related to immune system functioning. Another epigenome-wide study compared blood DNA methylation profiles between institutionalized children and children who were raised by their biological parents when the children were 7–10 years old (Naumova et al. 2012). 914 CpG sites were differentially methylated with most sites showing increased DNA methylation in institutionalized children as compared to the noninstitutionalized children. Subsequent clustering of all children based on these 914 CpG sites could reliably categorize individuals to one of the two groups, suggesting distinct DNA methylation profiles of these CpGs for institutionalized versus noninstitutionalized children. Differentially methylated regions were found in genes involved in the control of cellular signaling systems and the immune response.

Parental Stress Maternal stress during infancy and paternal stress during the preschool period were associated with DNA methylation profiles in buccal epithelial cells of mid-adolescent offspring (Essex et al. 2013). Notably, differentially methylated regions showed mainly increased DNA methylation status in adolescents exposed to parental stress. Additionally, while maternal stress was related to DNA methylation profiles in girls and boys, paternal stress was more strongly related to DNA methylation in girls.

To conclude, the parental environment – including parental care, parental loss, parental separation, and parental stress during the childhood years – is associated with changes in DNA methylation profiles across multiple genes implicated in HPA axis regulation, the serotonergic system, immune function, and neuronal plasticity. Furthermore, these epigenetic adaptations can even be observed in adulthood.

11.4.3 Socioeconomic Status and Other Psychosocial Factors

Socioeconomic status and other psychosocial factors outside the family environment play an important role for a healthy child development. For example, an adverse socioeconomic environment has been associated with cardiovascular,

respiratory, and heart diseases, stroke, cancer, and overall mortality (Galobardes et al. 2004). This association might be mediated by adaptations in biological systems and by long-lasting regulation of gene activity (Miller et al. 2009).

Socioeconomic Status Epigenetic profiling of promoter regions across the methylome in peripheral blood cells revealed differential DNA methylation between high and low early socioeconomic status at three CpG sites (Lam et al. 2012). When individuals were grouped based on their similarity of DNA methylation profiles, one of the groups consisted of a high proportion of individuals with low socioeconomic status. Furthermore, blood DNA methylation differed across 1252 gene promoter regions between men growing up in an extremely low and a particularly high socioeconomic background during childhood (Borghol et al. 2012). Socioeconomic status in adulthood was associated with DNA methylation in 545 mostly non-overlapping gene promoters, suggesting distinct blood DNA methylation profiles related to childhood socioeconomic status in middle-aged men. Many differentially methylated regions were located on chromosomes 5, 11, and 19 in a bidirectional manner (low childhood socioeconomic status associated with greater DNA methylation in chromosomes 5 and 11 and lower DNA methylation in chromosome 19, respectively). Differentially methylated regions were overrepresented in the MAPK (mitogen-activated pathway kinase) and the functionally related chemokine pathway, which are involved in cellular differentiation, -growth and apoptosis, as well as in immune cell migration (Le et al. 2004; Santarpia et al. 2012). In another study, low family income and being raised by a single parent predicted higher DNA methylation in spermidine/spermine N1-acetyltransferase family member 2 (*SAT2*) and Alu repetitive elements, used as indicators of global DNA methylation, in peripheral blood granulocytes of a female-only sample (Tehranifar et al. 2013). Lastly, increased cumulative socioeconomic stress, such as financial hardship, low parental education levels, and single parent household, was associated with *SCL6A4* exon 1 methylation in carriers of the *SLC6A4* short risk allele; this effect was more robust in women (Beach et al. 2014).

Peer Environment Twin studies revealed differences in *SLC6A4* methylation of bullied twins compared to their non-bullied twin at 10 years of age (Ouellet-Morin et al. 2013). Compared to their non-bullied twin, DNA methylation in *SLC6A4* increased from 5 to 10 years, the period during which the bullying occurred. The increase in *SLC6A4* methylation was accompanied by an attenuated cortisol reaction in response to exposure to a distinct psychosocial stressor, the Trier Social Stress Test (TSST).

In conclusion, socioeconomic status and an adverse peer environment during child development seem to be linked to DNA methylation profiles in stress-related genes, such as *SLC6A4*, and in stress-related biological pathways, including gene clusters involved in the immune system and cell development. These epigenetic profiles are probably distinct to the early socioeconomic environment and independent from socioeconomic adversities in adulthood.

11.5 Chronic and Lifetime Stress and DNA Methylation

The detrimental effects of severe psychosocial stress are not limited to the childhood years. In adulthood, the psychosocial environment can contain sources of stress, ranging from mild daily hassles to chronic job stress to traumatic experiences. Exposure to chronic stress can deplete an individual's coping and energy resources, resulting in exhaustion and mental health problems (Meinlschmidt et al. 2009). Likewise, exposure to a single or repeated severe traumatic event, such as sexual violence, war experience, or other distressing events, can create an extraordinary burden and increase the risk to develop a mental disorder. Investigating the association between chronic psychosocial stress during adulthood and epigenetic patterns in genes of the stress system may shed light on epigenetic processes associated with the risk for the development of stress-related mental disorders beyond childhood. Since epigenetic adaptations related to trauma exposure during adulthood are discussed in Chap. 6, we will focus on more chronic daily stressors in this chapter. All results are summarized in Table 11.1.

11.5.1 Psychosocial Stress in Adulthood

Chronic or severe stress exposure in adulthood is a risk factor for physical and mental disorders alike (Meinlschmidt et al. 2009). A limited number of studies focusing on perceived stress and DNA methylation in adulthood suggest that stress-related alterations in the epigenome are not restricted to sensitive periods during development. For instance, healthy adults carrying the Val/Val polymorphism in a catechol-o-methyltransferase (*COMT*) SNP and reporting greater levels of stress had decreased DNA methylation at this particular SNP in peripheral blood cells as compared to Val/Val carriers with reduced levels of stress (Ursini et al. 2011). There were no differences in an examined *COMT* promoter region or a *LINE*-1 (long interspersed nuclear element). Increased DNA methylation in the *COMT* SNP region predicted lower *COMT* expression and higher accuracy in a working memory task. Additionally, lower *COMT* methylation and greater stress levels were associated with increased and thus less efficient prefrontal activity in the working memory task. These results suggest that DNA methylation, as measured in the periphery, might be related to brain activity and could therefore act as an indicator of COMT activity in the brain. Indeed, a subsequent rat experiment indicated that DNA methylation in a CpG site corresponding to the Val/Met polymorphism in humans was correlated between peripheral mononuclear blood cells and the prefrontal cortex but not the hippocampus or striatum (Ursini et al. 2011). Remarkably, these results illustrate how psychosocial stress and genotype interact to predict phenotype, more specifically working memory performance and brain activity.

Similarly, nurses under high work stress condition reported elevated symptoms of burnout and had lower DNA methylation levels in five investigated CpG sites in

the *SLC6A4* promoter region measured in peripheral blood samples as compared to nurses experiencing low work stress (Alasaari et al. 2012). Work stress and suffering from burnout symptoms but not work control, work demand, age, or carrying the short risk allele in *SLC6A4* were associated with weighted mean DNA methylation in the investigated *SLC6A4* promoter region. Notably, while work stress was related to decreased DNA methylation, burnout was associated with an increase in DNA methylation. This finding suggests bidirectional epigenetic adaptations related to stress and mental well-being and might indicate that nurses who were susceptible to stress showed the opposite epigenetic changes as compared to resilient nurses. In contrast, Wankerl and colleagues (2014) did not find any stress-related changes in *SLC6A4* methylation across an entire CpG island in healthy adults. Finally, adult subjects with low socioeconomic status and manual workers showed global DNA hypomethylation as compared to individuals with a high socioeconomic status and nonmanual workers (McGuinness et al. 2012).

11.5.2 Current Levels of Stress in Studies on Early Life Adversity

Several studies on early life adversities also assessed current levels of perceived stress. In the study by Tyrka and colleagues (2012) on childhood adversities and *NR3C1* methylation in healthy adults, current levels of perceived stress and attenuated cortisol response related to exposure to a combined dexamethasone/CRH test were associated with increased DNA methylation of CpG sites neighboring the *NR3C1* exon 1_F NGFI-A binding site. Similarly, greater DNA methylation in one CpG site located in the promoter region of *SLC6A4* (Kang et al. 2013) was linked to increased levels of current subjectively reported stress in depressed patients. Additionally, increased average DNA methylation in the *SLC6A4* promoter region was linked to a reduced level of social and occupational functioning and quality of life and greater disability (Kang et al. 2013). Finally, current levels of perceived stress and salivary cortisol predicted epigenome-wide patterns of DNA methylation in peripheral blood of healthy adults with high or low childhood socioeconomic status (Lam et al. 2012).

Taken together, the findings from studies on current levels of stress and DNA methylation propose changes in the methylome, which might point out the possibility of stress adaptation on an epigenetic level beyond childhood. However, the mechanisms underlying these associations, for example as pointed out by Klengel and colleagues for *FKBP5* (2013), remain to be scrutinized in future studies. Moreover, for the studies not including early life adversities in their analysis, it is unclear whether the stress-related epigenetic modifications are a result of early life adversities or current stress levels in adulthood. Specifically, stress exposure during early development might program the stress response (Lupien et al. 2009), which could result in higher levels of stress later in life.

11.6 Acute Stress Response and DNA Methylation

Although research has addressed biological and psychosocial processes related to the acute stress response extensively, epigenetic studies are still scarce. For instance, our group found dynamic changes in DNA methylation in two *OXTR* regions related to exposure to the TSST (Kirschbaum et al. 1993) in adult participants who grew up during and shortly after World War II (Unternaehrer et al. 2012). More specifically, we found an increase in DNA methylation of one target sequence in the *OXTR* from pre-stress to immediately after stress and a decrease from immediately after stress to a 90 minute recovery period, with DNA methylation status even below baseline level. This DNA methylation decrease from post-stress to recovery was also found in a second *OXTR* target sequence. Although some of these dynamic changes might be mediated by shifts in blood cell count, the decrease from post-stress to recovery was independent from blood cell distribution. A second study on dynamic DNA methylation investigated a target region in perforin 1 (*PRF1*), which is involved in cytotoxicity and the immune system, before and after exposure to the TSST in study participants with and without chronic fatigue syndrome (Falkenberg et al. 2013). Remarkably, DNA methylation in all seven CpG sites examined in *PRF1* was increased after the stress exposure in the entire sample, although more pronounced in non-fatigue individuals. Moreover, average *PRF1* methylation before the TSST was correlated with *PRF1* expression levels across multiple time points, while post-TSST *PRF1* methylation was correlated with expression in non-fatigue subjects only.

To conclude, despite the need for replication, these studies indicate that DNA methylation might change during short-term stress adaptation operating within a few hours after psychosocial stress exposure. Scrutinizing dynamic stress-associated changes might prove to be essential for understanding the differences in stress reactivity in healthy subjects and individuals suffering from mental disorders. Thus, future research needs to address the dynamics of epigenetic modifications related to psychosocial experiences and the stress response, for example, whether epigenetic alterations occur within days, hours, or minutes after a distinct period of psychosocial stress.

A second highly relevant question is whether distinct epigenetic patterns can predict the magnitude or duration of the stress response. For instance, Edelman and colleagues (2012) found that DNA methylation of *NR3C1* in DNA extracted from buccal cells predicted total stress-related cortisol output in healthy female, but not male, university students who underwent the TSST. Although an independent study applying an alternative psychological stressor reported similar results – associations of lower blood *NR3C1* methylation with attenuated stress response as indicated by heart rate, decreased cortisol output and lower perceived stress, and decreased performance and feelings of control – they found that these associations could be mainly explained by education and lifestyle (de Rooij et al. 2012). In contrast, a recent study applying the TSST found that the interaction between DNA methylation in an *SLC6A4* CpG island and *SLC6A4* genotype predicted cortisol stress response in young healthy adults (Alexander et al. 2014): participants with low

SLC6A4 methylation showed an association between *SLC6A4* genotype and cortisol stress reactivity, in a dose-dependent manner, with carriers of the short risk allele having an increased stress response. No similar effect was found for participants with high *SLC6A4* methylation. These finding suggests that DNA methylation status and genotype interact to predict phenotype.

Further studies on acute psychosocial stress and DNA methylation are warranted, especially with regard to the dynamics of DNA methylation in response to psychosocial stress. The finding that DNA methylation might predict stress reactivity is promising and underlines the need for future studies.

11.7 Future Perspectives and Implications for Research and Clinical Practice

A range of studies suggests that prenatal and early life experiences program the epigenome, including genes involved in HPA axis or the serotonergic and immune system. This might have consequences for gene expression and protein synthesis in these biological systems and result in variation in stress adaptation, susceptibility, or resilience for mental disorders. Nevertheless, multiple issues remain to be addressed in future research on epigenetic programming by psychosocial stress experiences:

The first issue is whether epigenetic changes are observed after exposure to psychosocial stress per se or whether epigenetic modifications are restricted to individuals who are already susceptible to develop or suffer from mental disorders? Or, on the contrary, are epigenetic modifications more pronounced in resilient individuals promoting an increased potential for stress adaptability? Comparing DNA methylation profiles after psychosocial stress exposure between subjects suffering from mental disorders and healthy individuals with comparable experiences might promote the identification of epigenetic biomarkers. Ideally, these markers could have clinical relevance, for example, by facilitating the identification of individuals at risk for developing a mental disorder after exposure to psychosocial stress.

A second pertinent question comprises epigenetic modifications associated with positive experiences or health-promoting interventions, such as psychotherapy. Notably, a few studies reported changes in DNA methylation in patients responding to psychotherapeutical treatment versus nonresponders. Specifically, *BDNF* methylation was decreased after successful dialectic behavioral therapy treatment in patients with borderline personality disorder as compared to increased *BDNF* methylation in nonresponders (Perroud et al. 2013). Furthermore, combat veterans suffering from PTSD who underwent exposure-based psychotherapy showed changes in *FKBP5* exon 1 methylation after the treatment as compared to pretreatment: patients with symptom improvement showed decreased *FKBP5* methylation levels, in contrast to increased levels after treatment in nonresponders (Yehuda et al. 2013). In addition, pretreatment *NR3C1* exon 1_F promoter methylation predicted treatment

response but showed no treatment-related alterations in DNA methylation. These results support the hypothesis that psychotherapy might reverse adverse epigenetic profiles in stress-related candidate genes. Notably, a study by Day and colleagues (2013) suggested that DNA methylation of *Egr1* and *Fos* might be involved in reward learning in rats. Moreover, animal studies demonstrated epigenetic alterations related to enrichment (Sweatt 2009; Branchi et al. 2011) or exercise (Gomez-Pinilla et al. 2011). Whether similar DNA modifications apply for positive events in humans remains to be elucidated in future studies.

A third problem is whether epigenetic patterns are influenced by psychosocial stress or whether they are a pure correlate of these experiences. Although prospective longitudinal studies might provide clues on causal pathways, they cannot provide information on causal relationships. While a few animal experiments applying a cross-fostering design suggest that the early environment might be the cause of the epigenetic modifications (Weaver et al. 2004), similar research in humans is restricted by ethical considerations. Investigating the human epigenome across multiple time points and adoption studies are necessary to shed light on this issue.

The fourth issue, particularly in human studies, is the identification of epigenetic effects of a distinct environmental factor, violence, neglect, parental psychopathology, low socioeconomic status, low education, or life stress, which often coincide and are associated with a higher prevalence of physical and mental disorders, probably in a cumulative manner (Scher et al. 2004; Appleyard et al. 2005; Hussey et al. 2006; Sellström and Bremberg 2006; Melchior et al. 2007).

Another major concern is the translation of epigenetic patterns derived from peripheral tissue, such as peripheral blood or buccal epithelial cells, to neuronal tissue. A few studies linking DNA methylation in the periphery to brain activity (Ursini et al. 2011; Connelly and Morris 2012; Puglia et al. 2015; Frodl et al. 2015) and studies examining DNA methylation profiles across multiple tissue (Byun et al. 2009; Davies et al. 2012; Ma et al. 2014; Nemoda et al. 2015) suggest that DNA methylation at some genomic regions might be conserved across different tissues and that epigenetic patterns in the periphery might serve as a proxy for epigenetic patterns in the brain and could be related to brain activity. Despite the desire to translate epigenetic findings from the periphery to the brain, it is important to acknowledge that epigenetic profiles in peripheral tissue might provide relevant information about processes in the periphery. For instance, GR signaling in peripheral blood cells is impaired after chronic stress experience with consequences for immune functioning and subsequent stress-related morbidity (Miller et al. 2008).

Finally, epigenetic studies looking at stress-related epigenetic processes beyond DNA methylation, such as histone modifications or microRNAs, which have been implicated in neurodevelopmental disorders (Millan 2013) are scarce, especially in humans. For example, stress-related modification of histone tails has been reported in animal models (Desplats 2015). These additional epigenetic processes might contribute to a comprehensive understanding of epigenetic programming by psychosocial experiences. Furthermore, additional measures beyond average or single CpG DNA methylation, for example, variance or synchronization across CpG sites across a given region, different genes, or different tissues, could provide additional information on stress-related epigenetic adaptations.

In sum, several studies indicate changes in the epigenome related to psychosocial stress. Nevertheless, many of the here discussed studies should be replicated in independent samples; the sample sizes are often small and not representative of the target population, which leads to heterogeneous findings and inconclusive results. Furthermore, the upcoming research field of epigenetics might profit from quality standards, for example, as required for microarray experiments (Minimum Information About a Microarray Experiment, MIAME).

11.8 Conclusions

Psychosocial stress, particularly during early development, is a widely acknowledged risk factor for mental disorders. Epigenetic mechanisms provide a potential mediator in this association, whereby psychosocial stress "programs" the epigenome, with consequences for mental health. Taken together, human studies suggest a life-long sensitivity of the methylome for psychosocial experiences, starting from in utero exposure to maternal psychosocial stress and mood disturbances, to the childhood psychosocial environment, until adulthood and older age. Moreover, stress-related epigenetic alterations are not limited to specific candidate genes or CpG sites but can be observed across broad regions of the human methylome. Hence, DNA methylation and potentially additional epigenetic processes are promising molecular mechanism linking the psychosocial environment to the genome, with relevance for the development of stress-related disorders.

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