Epigenetics and Human Health

Arturas Petronis
Jonathan Mill *Editors*

# Brain, Behavior and Epigenetics

Springer

# Epigenetics and Human Health

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ISSN 2191-2262 e-ISSN 2191-2270
ISBN 978-3-642-17425-4 e-ISBN 978-3-642-17426-1
DOI 10.1007/978-3-642-17426-1
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2011929222

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*Cover design:* SPi Publisher Services

Printed on acid-free paper

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# Preface by the Series Editors

*Brain, Behaviour and Epigenetics* is the second volume of a new Springer book series on *Epigenetics and Human Health*, edited by Robert Feil (University of Montpellier, France), Mario Noyer-Weidner (University of Berlin, Germany) and Jörn Walter (University of Saarbrücken, Germany).

With Arturas Petronis and Jonathan Mill the series has attracted two editors to this volume with a long-standing record in this emerging biomedical field. They both direct brain- and disease-oriented research programs at the Centre for Addiction and Mental Health in Toronto (Canada) and at the Kings College in London (UK), respectively. In the 14 chapters of their book they have gathered comprehensive articles ranging from basic molecular regulation in the brain to memory formation and psychiatric disorders.

The series adds to Springer's growing interest in the field of epigenetics and its many links to human health – both in the biological and clinical context. The aim of the book series is to introduce new epigenetic topics and concepts with relevance to human disease and to present them in a broad interdisciplinary context. Along this line the series aims to address both clinicians and basic scientists. We very much hope you will enjoy this volume and the series and find them useful for your professional endeavours.

May 2011

Robert Feil
Mario Noyer-Weidner
Jörn Walter

# Preface to the Volume

Biomedical research in the first decade of the twenty-first century has been marked by a rapidly growing interest in epigenetics. The reasons for this are numerous, but primarily it stems from the mounting realization that research programs focused solely on DNA sequence variation, despite their breadth and depth, are unlikely to address all fundamental aspects of human biology. Some questions are evident even to nonbiologists. How does a single zygote develop into a complex multicellular organism composed of dozens of different tissues and hundreds of cell types, all genetically identical but performing very different functions? Why do monozygotic twins, despite their stunning external similarities, often exhibit significant differences in personality and predisposition to disease? If environmental factors are solely the cause of such variation, why are similar differences also observed between genetically identical animals housed in a uniform environment? These are not necessarily new questions. More than half a century ago, Conrad H. Waddington, an insightful British developmental biologist, developed a theoretical framework to explain how identical genotypes could produce a wide collection of phenotypes over the course of development, defining epigenetics as the developmental processes "connecting" a cell's genotype to its phenotype. Even before Waddington, the term *epigenesis* was used several centuries ago to conceptualize how, starting from a uniform material, every individual acquires new forms that emerge gradually over time.

Over the last couple of decades, epigenetics has undergone a significant metamorphosis from an abstract developmental theory to a very dynamic and rapidly developing branch of molecular biology. Waddington's concept of an "epigenetic landscape" linked to phenotype has materialized into the study of complex combinations of DNA and histone modifications, together acting to coordinate various genetic functions in the cell. These modifications can be heritable, both mitotically and meiotically, but do not involve changes in the DNA sequence. Contemporary epigenetics investigates DNA methylation and hydroxymethylation, in addition to a plethora of histone modifications that together play a critical role in a variety of regulatory processes within the nucleus. Epigenetic processes primarily regulate gene expression, controlling the tissue-specific orchestration of gene activity that ultimately accounts for the development of multicellular organisms. They also have a number of other important genomic functions including the suppression of

retroelements, the instigation of X chromosome inactivation in females and a role in meiotic and mitotic recombination and chromosomal segregation.

Epigenetic studies in various species – from *Escherichia coli* and yeast to animals and humans – are now underway, highlighting how epigenetic regulation is critically important for the normal functioning of the genome. Cells can operate normally only if both DNA sequence and epigenetic components of the genome function properly. In other words, the cell needs both the DNA "hardware" and the epigenetic "software." Furthermore, epigenetic factors and the DNA sequence do not necessarily operate in isolation. Some DNA sequence variants can influence local epigenetic profiles, for example, via processes such as allele-specific DNA methylation. Likewise, epigenetic modifications can predispose certain nucleotides to be more mutagenic than others; for example, methylated cytosines are prone to spontaneous deamination and conversion to thymines.

Epigenetic information varies among cells of the same tissue, across the cells of different tissues, and also between individuals. Epigenetic changes may be rapid and short-lived (e.g., the cyclical changes observed in response to the circadian rhythm) or highly stable once established (e.g., the maintenance of tissue and cell type specificity). Despite the extremely fast growth in epigenetic research over the last decade, we are only just beginning to understand the full complexity of the epigenome. We are still mapping the epigenetic landscape of the cell, uncovering new epigenetic mechanisms, and novel roles for epigenetic processes. Recent years, for instance, have seen the recognition about the importance of noncoding RNA and RNA-mediated epigenetic gene regulation, as well as physical interactions among genes in three-dimensional space within the nucleus.

The recent advent of high-throughput genomic approaches, first via the application of microarrays and more recently via next generation sequencing, has enabled a technological quantum leap in molecular epigenetic studies; epigenome-wide mapping experiments can now be feasibly performed at unprecedented resolution. The term "epigenome" is used to describe the complex distribution of epigenetic modifications across the entire genome in a specific cell or cell population. As in genome-wide association studies, scans of epigenomes will soon become a routine procedure in experimental laboratories. Large-scale epigenomic mapping projects initiated by the NIH, European Science Foundation, and other major funding agencies have already mapped numerous layers of epigenomic information using these latest technologies. Hopefully, this effort will result in a global, integrated view of different cellular states. Unlike the human genome, mapping the epigenome is an open-end project: each cell in each individual may have a distinct epigenome that reflects its developmental state, environmental exposures, stochastic effects, among numerous other multidirectional effects that form the epigenetic uniqueness of each cell, each tissue, and each individual.

The focus of this volume is behavioral and brain epigenetics, representing a novel a frontier in neurobiological and psychiatric research. One of the primary objectives of behavioral epigenetics is to understand the molecular basis of various brain functions (e.g., memory, cognition, homeostasis, and adaptation to new environments). Of particular interest is the putative role of epigenetic dysfunction in brain pathology

and mental illness. While epigenetic factors have been intensively investigated in the malignant transformation of cells in cancer, similar processes may be highly relevant to various complex non-Mendelian diseases. Epigenetic mechanisms – often more efficiently than genetic ones – are able to integrate a number of apparently unrelated clinical, epidemiological, and molecular data into a new theoretical framework. Putative epigenetic misregulation is consistent with various epidemiological, clinical, and molecular features in complex diseases, including most psychiatric disorders. It is apparent that the dysregulation of gene activity and deviations from a normal expression pattern can be as detrimental to a cell as mutant DNA sequences resulting in dysfunctional proteins. It is important to note that epigenetic changes that are partially both inherited and acquired can be the primary disease causes, rather than just one of numerous secondary or further downstream epiphenomena.

Another pertinent question is how exposure to a wide scope of environmental factors, such as toxins, drugs of abuse, infection, nutrition, and stress can affect epigenetic regulation in the brain that ultimately translate into alterations in behavior. Epigenetic studies will provide new insights into the interface between the environment and the genome, and the mechanisms by which exposures at key points in development may mediate long-term effects on behavior. Some epigenetic changes are transient, whereas others may be relatively stable and persist much longer. Some chromatin changes are mitotically heritable and can affect somatic tissues, whereas others may even be inherited through meiosis and affect subsequent generations.

This volume represents a compilation of our current understanding about the key aspects of epigenetic processes in the brain and their role in behavior. The chapters in this book bring together some of the leading researchers in the field of behavioral epigenetics. They explore many of the epigenetic processes that operate or may be operating to mediate neurobiological functions in the brain and describe how perturbations to these systems may play a key role in mediating behavior and the origin of brain diseases. Akbarian et al. analyze the mechanisms by which epigenetic factors, such as covalent histone modifications, can contribute to dysregulation of gene expression in schizophrenia. Another chapter dedicated to schizophrenia, by Grayson and colleagues, summarizes their detailed epigenetic analysis of genes encoding reelin and glutamate decarboxylase 67. Theoretical evidence, with some preliminary experimental findings, that bipolar disorder is a promising candidate for epigenetic and epigenomic studies is summarized by Kato. The epigenetics and disease theme is further elaborated by Labonté and Turecki, who discuss the complex relationship between adverse life events and social stress, on the one hand, and major depression and suicidal behavior on the other, exploring the putative role of molecular epigenetic factors. Malaspina et al. provide epidemiological evidence that paternal age, toxin exposure, and psychological stressors may increase the risk for mental disease and suggest that these environmental hazards can be indirectly uncovered using epigenetic strategies, both in humans and animal models. The theme of epidemiological epigenetics in psychiatric diseases is elaborated by Susser et al., who discuss how prenatal famine and childhood ethnic minority status is associated with higher degree of psychopathology. Craig et al. discuss the complex epigenetic regulation of the X chromosome and its impact on human behavioral

phenotypes. Crespi, in a theoretical *tour de force*, attempts to link parent–offspring conflict, attachment theory, and genomic imprinting. The numerous roles of genomic imprinting, a classical epigenetic mechanism, in brain and behavior studies are overviewed by Isles and Wilkinson. The intriguing observations about the epigenetic effects of social experiences occurring during infancy, and its role in the establishment and maintenance of environmental programming are summarized in two chapters, one by Curley and Champagne and a second by Weaver. Finally, three chapters by Labrie, Estevez and Abel, and Reul et al. discuss evidence that epigenetic mechanisms can play a critical role in synaptic plasticity, learning, and memory.

Despite significant progress in molecular epigenetic research and its enormous potential, there are still considerable challenges to overcome before we can fully understand the role of epigenetic processes in brain function and behavior. For instance, what comprises a "normal" brain epigenome and what is the degree of tissue and cellular specificity of epigenetic landscapes in the brain? How do the multiple layers of epigenetic information interact and change over time? How common is meiotic epigenetic heritability and what role it may play in complex psychiatric disease? To what extent is the epigenome plastic and malleable in response to environmental influences? This volume demonstrates that such questions can now be explored in an experimental molecular biology laboratory. While the community is only just starting to acknowledge the importance of epigenetic processes in the brain, there is no doubt that numerous breakthrough discoveries in brain and behavioral epigenetics will be made in the decades to come.

Toronto, Canada Arturas Petronis
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# Chapter 1
# Posttranslational Histone Modifications and the Neurobiology of Psychosis

**Schahram Akbarian, Iris Cheung, Caroline Connor, Mira Jakovcevski, and Yan Jiang**

**Abstract** Schizophrenia and related major psychiatric disease is typically defined by the conspicuous absence of a defining neuropathology and a lack of straightforward identifiable genetic factors in the majority of affected individuals. On the other hand, there is increasing evidence that a distinct set of RNAs, many of which encode proteins of critical importance for myelin regulation and oligodendrocyte function, or GABAergic inhibitory and glutamatergic excitatory neurotransmission are expressed at altered levels in diseased brain. This chapter explores the mechanisms by which epigenetic regulators of gene expression, including covalent histone modifications, could contribute to dysregulation of gene expression in schizophrenia. There is also discussion on the methodological and scientific limitations of histone-focused approaches, as it pertains to the human (postmortem) brain, as well as brief remarks on the topic of epigenetic heritability of chromatin structures potentially altered in schizophrenia. The authors predict that the study of histone modifications, both at defined candidate gene loci and genome-wide, will become an important tool in the investigation of gene expression abnormalities and potential epigenetic dysregulation in the brains of subjects on the psychosis spectrum.

**Keywords** Epigenetics · Heritability · Histone code · Nucleosome · Schizophrenia · Transcriptional regulation

## Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| DRD2 | Dopamine receptor D2 |
| ERBB4 | Receptor tyrosine kinase erb4 |

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_1,
© Springer-Verlag Berlin Heidelberg 2011

| | |
|---|---|
| GABA | Gamma-aminobutryic acid |
| GABRA2 | GABA-A receptor, alpha 2 subunit |
| GAD1 | Glutamic acid decarboxylase 1 |
| GAD67 | 67 kDa glutamic acid decarboxylase (=GAD67) |
| GRIN1 | Glutamate receptor, ionotropic 1 |
| GRM3 | Glutamate receptor, metabotropic 3 |
| H3K4me1 | Histone H3-monomethyl-lysine 4 |
| H3K4me2 | Histone H3-dimethyl-lysine 4 |
| H3K4me3 | Histone H3-trimethyl-lysine 4 |
| HDAC | Histone deacetylase |
| HDACi | Histone deacetylase inhibitor |
| Hz | Hertz |
| K | Lysine |
| KDM | Histone lysine demethylase |
| KTM | Histone lysine methyltransferase |
| Mll1 | Mixed lineage leukemia 1 (histone methyltransferase) |
| mRNA | Messenger ribonucleic acid |
| NMDA | *N*-methyl-D-aspartate |
| SNP | Single nucleotide polymorphism |
| SUMO | Small ubiqutin related modifier |
| VPA | Valproic acid |

## 1.1 The Neurobiology of Schizophrenia

### *1.1.1 The Pathophysiology of Psychosis: Synopsis and Overview*

Schizophrenia is a complex neuropsychiatric disorder characterized by positive symptoms, including auditory and other sensory hallucinations and delusions; negative symptoms, such as social withdrawal and flat affect; and cognitive symptoms, including working memory deficits and disorganized speech and thinking. Symptoms typically emerge in young adulthood, and often lead to significant social and occupational dysfunction. The large majority of subjects diagnosed with schizophrenia or a related psychiatric disease do not demonstrate robust alterations in brain structure or morphology, nor do they show a defining cellular pathology in the CNS or elsewhere, giving rise to the view that disease-associated neuropathology, such as increased ventricular volume and small decreases in gray matter volume, is subtle (Harrison 1999; Iritani 2007).

Likewise, changes on the cellular level are relatively minor compared with many other neurological disorders. For example, decreased density and altered spatial distribution of oligodendrocytes in conjunction with myelin deficiencies have been shown in the gray and white matter of prefrontal cortex and hippocampus

(Hof et al. 2003; Schmitt et al. 2009; Uranova et al. 2004), suggesting that the brain connectivity is impaired. In addition, both increased numbers and distorted distribution of subcortical white matter neurons have been reported in the frontal, temporal, and parietal lobes (Akbarian et al. 1996; Connor et al. 2009; Eastwood and Harrison 2005; Ikeda et al. 2004; Kirkpatrick et al. 1999; Rioux et al. 2003). Altered distribution of neurons has also been demonstrated in layer II of entorhinal cortex (Falkai et al. 2000; Jakob and Beckmann 1986). These findings are thought to reflect compromised prenatal and early postnatal neurodevelopment, which are the critical periods for neuronal migration and positioning. Whether or not these histological alterations observed in clinical postmortem brain (Harrison 1999) are causally related to psychosis remains a matter of investigation.

Recently, in vivo neuroimaging and electrophysiological approaches have uncovered evidence for cortical dysfunction affecting portions of the forebrain, including prefrontal cortex and hippocampus, which often occurs in conjunction with cognitive deficits in the domains of working memory, executive control, and even some aspects of episodic memory (Barch 2005; Potkin and Ford 2009). One of the most exciting and more recent developments in the field concerns the study of neural oscillations and synchronization, which can be measured by electroencephalography and magnetencephalography. There is evidence that large cortical and subcortical neuronal networks operating in the beta (13–30 Hz) and gamma (30–200 Hz) range are abnormal in schizophrenia (Uhlhaas and Singer 2010). These widespread deficits in synchronization are believed to underlie some of the cognitive deficits observed in schizophrenia, including working memory and attention (Uhlhaas and Singer 2010). Although the precise pathophysiological underpinnings of these oscillatory abnormalities remain unknown, they are likely to result from a number of factors. For example, there is evidence of widespread changes in white matter integrity in schizophrenia (Lim et al. 1999; White et al. 2009), as well as the aforementioned deficits in oligodendrocytes (Hoistad et al. 2009) and white matter neurons beneath the cerebral cortex (Connor et al. 2009). These alterations may contribute to disordered connectivity between different cortical areas. In addition, multiple neurotransmitter systems have been implicated in the mediation of coordinated activity of neuronal assemblies (Uhlhaas and Singer 2010); in particular, deficits in inhibitory (GABAergic) local circuit neurons and their *N*-Methyl-D-Aspartate (NMDA) receptor-mediated excitatory (glutamatergic) input are thought to play a pivotal role in abnormal neuronal synchronization in psychosis (Belforte et al. 2010; Benes 2010; Homayoun and Moghaddam 2007; Lewis et al. 2008; Lisman et al. 2008; Sohal et al. 2009). The interrelation between these measures and biomarkers of cortical dysfunction with other abnormalities encountered in schizophrenia brain, such as the increases in striatal dopamine release after amphetamine challenge and at baseline, and in striatal baseline occupancy of dopamine $D_2$ receptors (Abi-Dargham et al. 2009), remains to be determined.

The above examples illustrate why many investigators in the field consider psychosis spectrum disorders to be maladies that are undoubtedly biological in

nature, but that are difficult to study due to the absence of a unifying and unambiguous morphological and cellular pathology.

### 1.1.2 Molecular Determinants of Cortical Dysfunction in Psychosis: Focus on RNAs and Proteins

It has become increasingly clear that proteomes and transcriptomes are abnormal in psychotic brain. For example, altered expression of oligodendrocyte proteins and mRNAs has been repeatedly reported in prefrontal and temporal cortex of schizophrenia subjects (Aston et al. 2004; Hakak et al. 2001; Katsel et al. 2005; Martins-de-Souza et al. 2009; Regenold et al. 2007; Tkachev et al. 2003). Likewise, many proteins and mRNAs encoding ligand-gated ion receptors, reuptake transporters, and metabolic enzymes for inhibitory GABAergic neurotransmission show altered expression in cerebral cortex in schizophrenia (Akbarian and Huang 2006; Benes 2010; Charych et al. 2009; Dracheva et al. 2004; Duncan et al. 2010; Guidotti et al. 2005; Hashimoto et al. 2008; Woo et al. 2008). In addition, transcripts for NMDA and other ionotropic glutamate receptors (Beneyto et al. 2007; Hemby et al. 2002; Meador-Woodruff and Healy 2000), as well as a variety of synapse- and other neurotransmission-associated proteins, and a diverse group of metabolic genes have been repeatedly implicated in schizophrenia postmortem brain studies (Khaitovich et al. 2008; Lewis and Mirnics 2006; Lipska et al. 2006; Middleton et al. 2002; Pongrac et al. 2004). It is important to emphasize that the above examples represent only an incomplete selection from a very large body of literature on mRNA and protein expression changes in postmortem schizophrenia and psychosis spectrum disorder brain, as a comprehensive list is beyond the scope of this chapter.

### 1.1.3 Altered Gene Expression in Schizophrenia Brain

Although no single gene transcript or protein has been consistently reported to be changed in schizophrenia or other psychosis spectrum disorder, the above-mentioned studies clearly point toward a select set of signaling pathways and molecules that are frequently altered in the brain of diseased subjects. The next question that arises regards the underlying mechanisms, which lead to altered mRNA levels. These are likely to reflect a complex and heterogeneous cache of primary causes, which can be grouped into two broad categories (1) genetic variation, including single nucleotide polymorphisms (SNPs) (Purcell et al. 2009) and structural variants, such as microdeletions and duplications (Kirov 2010) and (2) environmental factors, primarily adverse perinatal events, such as prenatal infection, maternal stress, and malnutrition (Kunugi et al. 2001; Lewis and Levitt 2002; Tandon et al. 2008). In addition, secondary factors that are not related to

disease pathogenesis could also result in gene expression changes; for example, antipsychotic medication has been shown to decrease the levels of oligodendrocyte-related mRNAs (Narayan et al. 2007). Furthermore, the physiological condition before death could potentially affect steady-state levels of metabolic and mitochondrial genes in the brain (Vawter et al. 2006).

However, the precise molecular mechanisms by which these primary and secondary factors exert their effects on mRNA levels – and the presumed downstream effects on brain function – remain unclear. Specifically, it is unknown whether these mRNA changes are due to a change in transcription or production, or rather due to altered metabolism or turnover. Many of the cellular mechanisms involved in these phenomena are, for technical reasons, difficult to study in human postmortem brain or even in preclinical models. Despite these limitations, there is increasing evidence that transcriptional regulation, or the mechanisms of gene expression, could play a role in the molecular pathology that involves some of the aforementioned disease-related mRNAs (Gavin and Sharma 2009; Shi et al. 2009). The caveat is that transcription is an extremely complex and dynamic process involving multiple steps (transcriptional initiation, postinitiation, elongation, termination, etc.) with multimeric transcription factor complexes, RNA polymerase subunits, and other molecules (Hager et al. 2009) that are difficult to study in their physiological context in the preclinical model, let alone in human postmortem brain.

One key aspect governing the regulation of transcription that is amenable for study in both animal models and in human postmortem brain is epigenetics, defined by chemical modifications of genomic DNA and the histone proteins (Berger 2007). Not surprisingly, the recent advances in epigenetics have greatly enhanced knowledge in preclinical models for depression, addiction, psychosis, and other neuropsychiatric disease (Abel and Zukin 2008; Buckley 2007; Duman and Newton 2007; Graff and Mansuy 2008; Renthal and Nestler 2008; Tsankova et al. 2007). In the following sections, we therefore provide an overview on covalent histone modifications, including their association with transcription. We also discuss some of the first examples that associate the regulation of posttranslational histone modifications with the neurobiology of psychosis.

## 1.2 Histone Modifications and the Molecular Pathology of Schizophrenia and Related Disease

### *1.2.1 Histones and the Postmortem Brain*

Histone proteins and their covalent modifications play a key role in the control of gene expression and genome organization. The fundamental structural unit of eukaryote chromatin is the nucleosome, a drum-like structure comprise an octamer of four core histones – H2A, H2B, H3, and H4 – wrapped with 147 bp of genomic DNA (Hayes and Hansen 2001; Wolffe 1992). Dynamic changes in chromatin

structure influence the accessibility of genomic DNA by transcription factors and are mediated by two interrelated molecular mechanisms: chromatin remodeling and histone tail modification (Felsenfeld and Groudine 2003). Chromatin remodeling is defined by the ATP-dependent mobilization of genomic DNA wrapped around the nucleosome core particle (Peterson 2002), and this process is tightly coupled with the histone tail modification machinery. Specific residues of the histone N-terminal tails are subjected to a variety of posttranslational and covalent modifications; these modifications exert their effects on chromatin structure and function via recruitment of a diverse array of chromatin binding proteins, resulting in either activation or repression of transcription. Specific combinations of these histone modifications working in concert to bring about a particular effect on transcription is referred to as the "histone code" (Berger 2002; Iizuka and Smith 2003; Jenuwein and Allis 2001; Peterson and Laniel 2004; Wang et al. 2004).

The acetylation of specific lysine residues in the N-terminal tails of H3 and H4 is associated with active gene expression, while deacetylation of these residues results in transcriptional silencing, chromatin condensation, and heterochromatin formation (Berger 2002; Iizuka and Smith 2003; Jenuwein and Allis 2001; Peterson and Laniel 2004; Wang et al. 2004). In addition, differential methylation, SUMOlyation, and ubiquitination of specific lysine residues, as well as phosphorylation of specific serine and methylation of selected arginine residues of H3 and H4 are associated with specific effects on transcription in eukaryotes, including humans (Berger 2002; Fischle et al. 2003; Iizuka and Smith 2003; Jenuwein and Allis 2001; Peterson and Laniel 2004; Wang et al. 2004). Of note, histone lysine residues can carry up to three methyl groups. These mono-, di-, and trimethylated forms of lysine residues show cell-specific regulation during development (Biron et al. 2004) and are differentially distributed across chromatin fibers (Bernstein et al. 2005). For example, trimethylated lysine residue 4 of histone H3 localizes to gene promoter regions, where it is associated with actual or potential transcription, whereas monomethylation of the same histone residue appears to be predominantly enriched at enhancer sequences further removed from transcription start sites (Heintzman et al. 2007) (Fig. 1.1). However, there is still significant overlap between the mono-, di-, and trimethyl mark of the same lysine residues (Barski et al. 2007). Multiplicity of function is not limited to the H3 lysine 4 residue, for example, the monomethylated forms of lysines 9 and 27 of histone H3 and lysine 20 of H4 are linked to gene activation, while di- or trimethylation of the same residues are associated with repression (Barski et al. 2007).

Recent postmortem studies provide evidence that histone modifications contribute to transcriptional regulation in the human brain (Ernst et al. 2009; Huang et al. 2006, 2007; Northcott et al. 2009; Stadler et al. 2005). In addition, recent preclinical studies demonstrated that the gene expression changes in animal models for learning or drug-induced plasticity is accompanied by dynamic alterations of histone tail modifications (Alarcon et al. 2004; Guan et al. 2002; Korzus et al. 2004; Kumar et al. 2005; Levine et al. 2005; Li et al. 2004; Swank and Sweatt 2001; Tsankova et al. 2004, 2006). Taken together, these studies suggest that chromatin remodeling and histone N-terminal tail modifications, which are traditionally explored in dividing

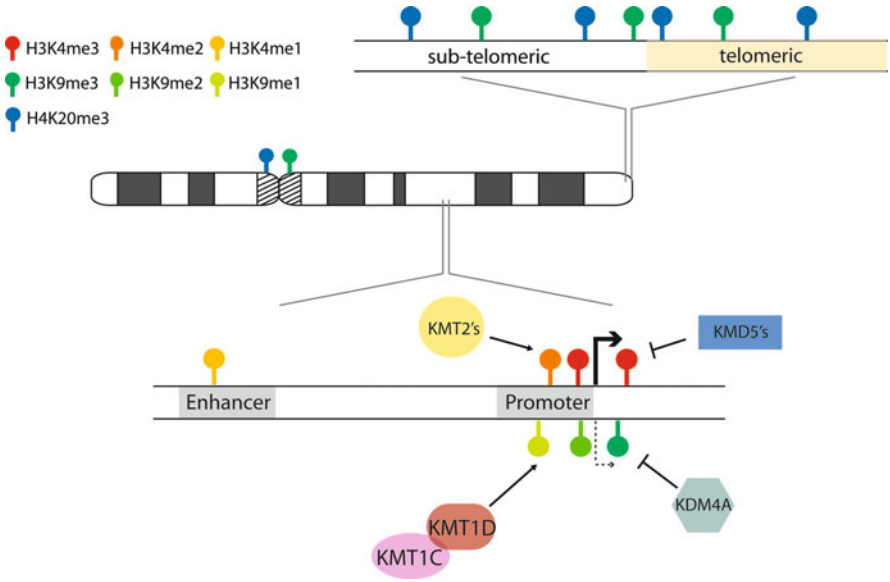


**Fig. 1.1** Differential regulation of site-specific histone modifications. The various chromosomal domains and chromatin states are associated with specific histone methylation patterns. Three specific histone lysine residues, H3K4, H3K9, and H4K20, are choosen as examples to illustrate this point. The monomethylated form of H3K4 (H3K4me1) is preferentially enriched at enhancer sequences, while the di- and trimethylated forms (H3K4me2 and H3K4me3, respectively) mostly define regions around transcription start sites. These marks are typically associated with open chromatin and show a rough correlation with gene expression levels. In contrast, H3K9 and H4K20 methyl markings are comparatively dense in constitutive heterochromatin, including pericentric and telomeric chromosomes and, when found at promoters, typically associated with negative regulation of transcription. Each of these marks is highly regulated by a specific cache of site-specific histone methyltransferases and demethylases. For example, the human genome encodes at least seven methyltransferases specific for H3K4 (KMT2A-H) and four demethylases having activities toward methylated H3K4 (KDM5A-D)

cells (Berger 2002; Iizuka and Smith 2003; Jenuwein and Allis 2001; Peterson and Laniel 2004; Wang et al. 2004), also play a critical role in regulation of transcription within the central nervous system and its postmitotic constituents, including neurons (Colvis et al. 2005; Jiang et al. 2008a; Martin and Sun 2004).

### *1.2.2 Histone Modification Changes in Diseased Brain: When Do They Occur?*

The origin of alterations in histone modifications in schizophrenic brain remains unclear. It is possible that histone modification changes occur very early during brain development, as the result of an innate, genetic abnormality, or by an external environmental stimulus. Indeed, the hypothesis that changes in histone modifications

reflect a stable and long-lasting epigenetic alteration in the brain of subjects with schizophrenia is currently the predominant theory in the field of psychiatric epigenetics.

A number of nonpsychiatric diseases, including diabetes, cardiovascular disease, and cancer, have been linked to environmental exposures during fetal development, and these exposures, in turn, have been found to cause epigenetic changes (Feinberg 2007; Gardner et al. 2007; Roberts 2010; Simmons 2009), leading to the hypothesis that many adult diseases with epigenetic underpinnings have fetal origins (Bezek et al. 2008; Doherty et al. 2009). Recently, it has been shown that certain prenatal environmental factors can influence the epigenetic programs in the fetal brain, and that these changes are detectable later in life. For example, adult mice prenatally exposed to cocaine have altered DNA methylation patterns in hippocampal neurons (Novikova et al. 2008). Environmental stimuli during early postnatal life have also been shown to influence epigenetic modifications in brain. One classic study linked the impact of maternal behavior during the first week of life with the altered DNA methylation at the glucocorticoid receptor in adult mouse brain (Weaver et al. 2004), demonstrating that the early postnatal environment has the capacity to influence adult behavior via induction of long-lasting epigenetic modifications.

Although evidence for heritability of epigenetic patterns is still limited, emerging data suggest that portions of the human epigenome may be derived from parental germlines, suggesting that altered histone modifications in schizophrenia could potentially be inherited. A recent study found that 4% of the haploid genome in sperm remains associated with nucleosomes (the majority of histones are replaced by protamine in sperm), and that H3K4me3, H3K4me2, and DNA hypomethylation were significantly enriched at the promoters of genes critical for early embryonic development (Hammoud et al. 2009), suggesting that parental genomes may transmit some epigenetic patterns to the offspring. Strikingly, a number of the genes associated with the H3K4me3 have been implicated in schizophrenia, including *GRIN1*, *GRM3*, *GAD1*, *GABRA2*, *ERBB4*, and *DRD2*. Alternatively, aberrant histone modification patterns in schizophrenia could potentially arise later in life, closer to the time of disease onset. Until recently, histone and DNA methylation were posited to be stable, irreversible epigenetic marks established during early development, allowing the generation and maintenance of unique cellular phenotypes (Klose and Zhang 2007; Ooi and Bestor 2008). In contrast, recent evidence derived from rodent and neuronal cell culture studies suggests that these modifications may, in fact, be reversible and capable of acute change in response to environmental cues (Dong et al. 2007; Tsankova et al. 2006). In addition, recent evidence indicates that – much like brain development itself – some epigenetic modifications, including histone and DNA methylation, continue to change in human brain during the progression from childhood to adulthood (Huang et al. 2007; Siegmund et al. 2007). Thus, it is possible that altered histone modifications arise due to the failure of normal "development" of epigenetic patterns in the brain during late adolescence or early adulthood, the typical period for disease onset.

In summation, additional studies will be necessary to clarify whether histone modification changes in schizophrenia postmortem brain represent stable imprints that exist for an extended period of time, or even precede the age at which clinical symptoms emerged in an affected individual.

### *1.2.3 Additional Limitations of Histone-Based Approaches*

An increasing number of clinical and translational studies, both in neuroscience and other fields of medicine exploring disease-related gene expression changes, are focusing on histone modification profiles at proximal promoters and other sequences critical for transcriptional regulation (Feinberg 2004; Feng et al. 2007; Li 2002; Maekawa and Watanabe 2007). In contrast to the more straightforward interpretations for any changes in expression of a specific gene transcript, it is important to realize that any alterations in histones and their modifications at a specific gene or gene promoter do not necessarily offer clear inference about potential changes in underlying gene expression activity.

The first problem that arises is rooted in the enormous degree of cellular heterogeneity of brain tissue, both in human and animal, with each anatomical area uniquely defined by highly heterogeneous mixtures of glutamatergic and GABAergic neurons and various types of glia. All these cells package their genome into the core nucleosome organization described earlier; thus, both cells that express a specific RNA and those that do not can potentially carry the same type of covalent modification at a specific genomic locus. In contrast to in situ hybridization histochemistry and other histological techniques, virtually all chromatin assays designed to map histone modification patterns lack single-cell resolution. Importantly, the majority of the preclinical and clinical work focused on detection of histone or DNA modification changes in neuropsychiatric disease is based on chromatin immunoprecipitation (ChIP) assays on brain tissue homogenate, as opposed to specific cell populations (Jiang et al. 2008b; Matevossian and Akbarian 2008). Therefore, most of these studies assume – but do not prove – that the specific cell population showing altered mRNA levels is also affected by the histone modification changes at the corresponding gene as observed in ChIP from tissue homogenate. This lack of cell resolution in ChIP assays is a significant limitation of the approach for any brain-related study, including some of the studies from the authors' laboratory (Huang and Akbarian 2007; Huang et al. 2007; Schroeder et al. 2008; Stadler et al. 2005).

A second limitation is based on the fact that specific histone modifications fulfill a range of functions (Berger 2007). A case in point concerns the H3-trimethyl-lysine 4 mark (H3K4me3), which primarily localizes to transcription start sites with a genomic occupancy pattern that roughly correlates with that of activated RNA polymerase II; moreover, there is some positive correlation between H3K4me3 levels at the 5′ end of a gene with levels of the corresponding transcript (Guenther et al. 2007). However, H3K4me3 is known to be a potential docking site for both activators and repressors of

transcription, and the actual effects on gene expression activity could follow either direction, depending on the presence or absence of specific chromatin remodeling complexes (Li et al. 2006; Shilatifard 2008), which can vary on the basis of the specific cellular context. Further complicating matters, H3K4me3 is not only a potential initiator of transcription but also a regulated event downstream from RNA polymerase II activity (Eissenberg and Shilatifard 2006). To avoid the issue that multiplicity of function results in ambiguity of interpretation for any histone modification changes observed in neuropsychiatric disease, it is probably best to probe the genome locus-of-interest for multiple types of histone modifications. Ideally, these should include marks associated with both open and repressed chromatin, and the combined histone modification profiles would be expected to provide important information about potential epigenetic changes in disease, including faulty repression or activation of specific gene loci. For representative examples in the field of translational neuroscience, see Stadler et al. (2005) and Tsankova et al. (2006).

### *1.2.4 Histone Modification Changes at GABAergic Gene Promoters During Normal Prefrontal Cortex Development and Alterations in Schizophrenia*

The following example illustrates how the study of specific histone modifications in schizophrenia brain, in combination with preclinical model systems, could potentially provide novel insights about the mechanisms of disease, even when taking into account the limitations mentioned in the previous section. It is generally accepted that subjects on the psychosis spectrum frequently show dysfunction and hypoactivity of the prefrontal cortex (PFC) (Goff and Evins 1998). Furthermore, it is often hypothesized that schizophrenia is associated with abnormal development of prefrontal and other neuronal circuitries (Harrison and Weinberger 2005). Notably, normal PFC circuitry does not reach full maturity until, or even after, adolescence and early adulthood (Fuster 2002; Segalowitz and Davies 2004). One interesting aspect of normal PFC development is that a substantial portion of the transcriptome shows a progressive increase in steady-state RNA levels during childhood and while transitioning from adolescence into young adulthood, potentially affecting many hundreds or thousands of gene transcripts (Harris et al. 2009; Somel et al. 2009). These include a number of GABAergic mRNAs, including the 67 kD form of glutamic acid decarboxylase (GAD67), a GABA synthesis enzyme (Colantuoni et al. 2008; Huang et al. 2007) that is frequently expressed at decreased levels in PFC and other cortical areas of adult subjects with schizophrenia (Akbarian and Huang 2006; Duncan et al. 2010; Impagnatiello et al. 1998; Torrey et al. 2005; Volk et al. 2000). Given this information, it is tempting to speculate that decreased GAD67 mRNA in adult subjects with schizophrenia reflects a failure to upregulate GAD67 gene expression in the developing PFC.

Indeed, it was recently reported that maturation of human and rodent cerebral cortex is associated with a progressive increase in methylation of H3K4 at GABAergic gene promoters. In the human PFC, GAD67 mRNA and promoter-associated H3K4me3 increased during the transitions from prenatal to childhood to adult stages (Huang et al. 2007). Furthermore, levels of H3K4me3 at a subset of GABAergic gene loci, including *Gad1* (the gene encoding GAD67), was dependent on normal gene dosage for Mll1 – an H3K4 specific histone methyltransferase expressed in GABAergic interneurons – in cortex of Mll1+/− mice (Huang et al. 2007). Interestingly, both H3K4 methylation and Mll1 occupancy were upregulated at the *Gad1* promoter after treatment with the atypical antipsychotic clozapine (Huang et al. 2007), a drug that improves working memory and other frontal lobe-associated cognitive functions (Meltzer 2004), suggesting that this epigenetic mechanism may play an important role in the underlying pathophysiology of schizophrenia. As a result, Mll1/MLL1-mediated histone lysine methylation at GABAergic gene loci emerges as a molecular link that interconnects three major factors in the neurobiology of psychosis: developmental mechanisms, interneuron dysfunction, and antipsychotic pharmacotherapy.

The Huang et al. (2007) study also illustrates some of the complexities faced by this field, as the disease-related decline in the GAD67 transcript and *GAD1* promoter-associated H3K4me3 levels was not a consistent feature in the clinical samples, but was dependent on a specific haplotype within a few kilobases from the *GAD1* transcription start site; furthermore, the effect appeared to be gender-specific. Notably, these same polymorphisms around the *GAD1* promoter were previously associated with schizophrenia and accelerated loss of frontal lobe gray matter (Addington et al. 2005; Straub et al. 2007). Interestingly, subjects with schizophrenia who are biallelic for the risk haplotype show not only decreased H3K4me3 but also increased levels of H3K27me3 (Huang et al. 2007), a mark regulated by repressive Polycomb group chromatin remodeling complexes associated with inhibition of transcription (Wang et al. 2004).

### *1.2.5 Chromatin and Antipsychotic Medication*

At present, very little is known about epigenetic effects of acute and long-term treatments with conventional (dopamine D2 receptor blocking) antipsychotics, or the various atypical antipsychotics, which are defined by a much broader receptor profile involving a range of dopamine and/or serotonin and other receptor systems. Table 1.1 provides a brief overview of mostly preclinical studies; based on this material, evidence is emerging that at least a subset of the atypicals, including clozapine as one of the most efficient antipsychotic drugs currently available, potentially induce histone acetylation and methylation changes in the cerebral cortex. In addition, D2-antagonist drugs induce various histone phosphorylation and phosphoacetylation (a dual modification defined by the phosphorylation of a serine residue in conjunction with acetylation of a neighboring lysine residue)

**Table 1.1** Histone modification changes after antipsychotic and mood stabilizer treatment

| Study | Histone (modification) | Medication effects |
|---|---|---|
| Stefanis and Issidorides (1976) | Nuclei staining with histone-sensitive dyes | Pimozide normalized chromatin staining patterns in white blood cells of patients with schizophrenia |
| Canoso and de Oliveira (1986) | Nuclear antibodies reacting with histone antigen | Chlorpromazine induces or raises serum antibody levels in patients with schizophrenia |
| Kumar et al. (1997) | Protein kinase C-catalyzed histone phosphorylatiion | Chlorpromazine mediated an inhibitory effect in the in vitro assay |
| Loncar-Stevanovic et al. (1998) | Chromatin accessibility | Chlorpromazine increased DNAseI susceptibility and chromatin degradation in isolated rat brain nuclei |
| Phiel et al. (2001) | H4 acetylation | Valproic acid upregulates acetylation in neuro2A cells |
| Li et al. (2004) | Histone H3-phosphoserine 10-acetyl-lysine 14 | Acute exposure to haloperidol is associated with increased phospho-acetylation in striatal neurons |
| Simonini et al. (2006) | H3 acetylation | Valproic acid upregulates acetylation in mouse hippocampus, striatum, prefrontal cortex |
| Sharma et al. (2006) | H3 and H4 acetylation | Valproic acid normalizes or upregulates acetylation in lymphocytes of bipolar and schizophrenia patients |
| Huang et al. (2007) | H3 lysine 4 trimethylation | Mice treated with clozapine show increased H3K4me3 at GAD1/GAD67 GABAergic gene promoter; postmortem brain (prefrontal cortex) of patients treated with clozapine show higher GAD1-associated H3K4me3 as compared with patients treated with typical antipsychotic |
| Dong et al. (2008) | DNA cytosine methylation | L-methionine-induced hypermethylation at the REELIN gene promoter is attenuated in frontal cortex of mice exposed to clozapine, sulpride, and valproic acid |
| Dong et al. (2008) | H3 lysine 9 and 14 acetylation | Increased in mouse frontal cortex after cotreatment with clozapine and valproic acid |
| Bertran-Gonzalez et al. (2008) | Histone H3 phosphorylation | Haloperidol-mediated increase in striatal neurons |

(*continued*)

**Table 1.1** (continued)

| Study | Histone (modification) | Medication effects |
|---|---|---|
| Leng et al. (2008) | Histone acetylation | In cerebellar granule cells, lithium enhanced the upregulation of acetylation and phosphorylation by several histone deacetylase inhibitor drugs, including valproic acid, sodium butyrate, trichostatin A, and panibinostat |
| Guidotti et al. (2009) | DNA cytosine methylation at Gad67 and reelin promoters | Clozapine and sulpride but not haloperidol decreased L-methionine-induced DNA hypermethylation in mouse frontal cortex |
| Guidotti et al. (2009) | H3 lysine 9 and 14 acetylation at Gad67 and reelin promoters | Increased in mouse frontal cortex after clozapine and sulpiride exposure |
| Bertran-Gonzalez et al. (2009) | H3 phospho-serine 10 and H3 phospho-serine 10-acetyl-lysine 14 | Haloperidol-mediated upregulation in striatopallidal medium spiny neurons |

*Chlorpromazine and thorazine*. Typical antipsychotics of the phenothiazine class. Antagonist for dopamine receptors D1, D2, D3, and D4; serotonin receptors 5-HT1 and 5-HT2, α1-, and α2-adrenergic receptors; and muscarinic acetylcholine receptors M1 and M2
*Chlozapine*. Atypical antipsychotic drug used for treatment of treatment resistant forms of schizophrenia. High affinity for the D4 receptor and partial 5-HT1A agonist
*Haloperidol*. Typical antipsychotic drug of the butyrophenone class used in psychotic episodes and in schizophrenia treatment. Strong D2 receptor antagonist
*Lithium*. In form of lithiumcarbonat the metal is used as a mood stabilizing drug to treat bipolar disorder, depression, and mania
*Pimozide or orap*. Antipsychotic drug of the diphenylbutylpiperidine class. Blocks strongly the D2 receptor, D3, α1-adrenergic, and 5-HT2A. Moderate blockade has been observed for D1, D4, α2-adrenergic receptors, and the dopamine transporter (DAT)
*Sulpride*. Antipsychotic drug used to treat schizophrenia and depression. Selective antagonist of postsynaptic D2 receptors
*Valproic acid*. Anticonvulsant and mood stabilizing drug. Used to treat seizures, bipolar disorder, and schizophrenia. Indirect GABA agonist by blocking the function of GABA transaminase. Some of the effects of VPA are due to its function as a histone deacetylase inhibitor (HDACi) leading to changes in gene expression. VPA is a classical HDACi, which blocks class I and II HDACs
*Panobinostat*. Experimental cancer drug, which works as a histone deacetylase inhibitor
*Sodium butyrate*. Classical histone deacetylase inhibitor (HDACi), which blocks class I and II HDACs
*Trichostatine A*. Histone deacetylase inhibitor (HDACi), which blocks class I and II

changes in striatum, which is an anatomical area in the forebrain densely innervated by dopaminergic fibers, and a key structure for the neuronal circuitry involved in psychosis. Currently, it remains unknown whether these changes are important for antipsychotic action or some sort of epiphenomena unimportant for treatment. Moreover, the molecular mechanisms mediating antipsychotic-induced (phospho) acetylation of striatal histones, and acetylation and methylation of histones in the cerebral cortex remain unknown. In contrast to the mood-stabilizing drug valproic

acid, which functions as a broadly acting histone deacetylase inhibitor both in intact brain and cultured cells, the histone methylation changes induced by the atypical clozapine require intact brain circuitry and are not apparent in neuronal culture (Huang and Akbarian 2007; Huang et al. 2007). Together, these findings would suggest that antipsychotic drugs could potentially interfere with the regulation of histone modification in brain cells, but these effects are likely to be indirect and unexplained by a direct action on specific chromatin remodeling proteins or histone modification enzymes. Meanwhile, there are also a few reports on antipsychotic-induced changes in white blood cell chromatin of patients, but the field, in general, has given little attention to these studies. To date, nothing is known about any chromatin-associated changes in the endocrine system of the pancreas and other organ systems implicated in metabolic syndrome, obesity and diabetes, which emerge as the most frequent side effects resulting from long-term exposure to a subset of atypical antipsychotics. Without question, epigenetic regulation, in the context of antipsychotic medication, is potentially a vital but largely unexplored topic.

**Acknowledgments** Work in the authors' laboratory is supported by grants from the National Institute of Health, the Deutsche Forschungsgemeinschaft, and the International Mental Health Research Organization (IMHRO).

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# Chapter 2
# Epigenetic Regulation of GABAergic Targets in Psychiatry

**Dennis R. Grayson, Marija Kundakovic, Ying Chen, Erbo Dong, and Alessandro Guidotti**

**Abstract** One of the more consistent findings observed in post mortem tissue from schizophrenia (SZ) patients is that the genes encoding reelin and glutamate decarboxylase 67 (*GAD67* or *GAD1*) are downregulated in cortex and other brain regions. Reelin is important for cortical migration during development and for synaptic plasticity and memory acquisition in the adult. GAD1 is one of two enzymes that synthesize the inhibitory neurotransmitter GABA in the central nervous system. Those neurons that make GABA are GABAergic and they serve a role in dampening excitatory neurotransmission throughout the brain. In addition, reports also show that NMDA receptor subunit expression and excitatory neurotransmission are reduced in cortical GABA neurons of SZ patients. Conditional knockout mice in which the NR1 subunit of the NMDA receptor is selectively reduced in GABA neurons of the brain show a downregulation of *GAD67* and parvalbumin (*PV*) mRNAs and also exhibit behaviors characteristic of SZ. These findings allow us to conceptually integrate two major schools of thought regarding the neurotransmitter deficit responsible for the symptoms of this psychiatric disorder. That is, if reduced glutamatergic neurotransmission occurs on GABAergic interneurons, the net effect would be reduced GABA output impacting the neuronal synchronization of pyramidal cell firing. Since it has also been shown that in GABAergic neurons, the mRNA encoding DNA methyltransferase 1 (*DNMT1*) is increased in SZ patients, this and other data suggest an epigenetic mechanism by which certain genes may be selectively downregulated contributing to SZ symptomatology. We propose that enzymes that methylate DNA and selectively reduce gene expression are hyperactive in patients with SZ and that this may be related to the pathogenesis of the disease. Here, we discuss these concepts in more detail and present our integrated view of synaptic transmission in SZ.

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_2,
© Springer-Verlag Berlin Heidelberg 2011

**Keywords** Cortex · DNA methylation · Gamma-aminobutyric acid (GABA) · Gene expression · Schizophrenia

## 2.1 Etiopathogenetic Mechanisms Underlying Schizophrenia and Bipolar Disorder

For several decades, monoamines (e.g., dopamine, serotonin, and norepinephrine) (Gray and Roth 2007; Hayman 2008; Lieberman et al. 2008) were the primary biochemical targets used to monitor molecular changes in the brain associated with psychoses. However, since the available antipsychotics that act on monoamine transmission fail to improve the negative and cognitive symptoms associated with psychosis, it is conceivable that an alteration of the monoaminergic system may not be the only source of the spectrum of clinical symptoms in SZ and bipolar disorder (BPD) patients. In fact, a compelling argument based on a comprehensive review of the literature suggests that dopaminergic signaling may represent the final common pathway impacted in SZ (Howes and Kapur 2009). Moreover, these authors suggest that future drug development should focus more on neurotransmitters such as GABA and glutamate, which act upstream of dopamine and that are likely to represent the primary defects associated with the disease. This concept was shaped in part by the notion that dopamine dysregulation, which is more closely linked with psychosis than to SZ, is associated with an altered release presynaptically rather than at the level of dopamine receptors. Presynaptic hyperdopaminergic release in particular brain regions is often linked to excessive excitatory receptor stimulation that is often modulated by GABAergic interneurons (Lisman et al. 2008).

Recent studies have identified abnormalities in GABA, glutamate, and acetylcholine neurotransmitter systems associated with SZ and BPD morbidity (Benes et al. 2007; Akbarian et al. 1995; Breese et al. 2000; Guidotti et al. 2000, 2005; Woo et al. 2005; Lewis et al. 2005). However, it is unclear whether these anomalies cause or are a consequence of these psychiatric disorders.

A number of factors may be responsible for the slow progress in identifying etiopathogenetic mechanisms underlying SZ and BPD. Factors that hamper progress in the discovery of satisfactory drug treatments include (1) the inability to identify alleles that confer increased risk for these diseases in the majority of patients, (2) the poorly understood neurobiological bases of cognition, emotion, and executive brain function that are disrupted in SZ and BPD; (3) the fact that SZ and BPD are diseases exclusively expressed in the human brain; thus any animal models will not represent all the symptoms and signs of the disease; and (4) the scarcity of well-validated accessible objective brain biomarkers that can reveal psychotic dysfunction in patients. The lack of objective testing means that diagnosis of SZ or BPD has to be made on the basis of phenomenological criteria established by clinical experts originating from the neuropsychiatric symptoms at presentation, subsequent drug responsiveness, symptom progression, and illness epidemiology.

Thus, this approach will not enable the identification of new drug targets or novel therapeutic mechanisms.

A recent theory is that these disorders may be the outcome of synergistic interactions between multiple susceptibility genes and environmental (neuroepigenomic) factors (Costa et al. 2007; Mellios et al. 2009; Mill et al. 2008; Ptak and Petronis 2008). However, this concept is still new and should be supported by studies identifying the underlying molecular events. Accordingly, the discovery of the primary epigenetic molecular mechanisms that induce downstream abnormalities of the multiple genes and neurotransmitters that are altered in brains of patients with SZ and BPD is essential.

## 2.2 Dysfunctional GABA/Glutamate Interactions in SZ and BPD

Several lines of evidence suggest that telencephalic GABAergic transmission may be defective in psychosis (Benes et al. 1992; Akbarian et al. 1995; Guidotti et al. 2000, 2005; Lewis et al. 2005). As a case in point, the expression of glutamic acid decarboxylase (*GAD67, GAD1*), reelin, *Reln*, *N*-methyl-D-aspartate receptor subunits (*GRIN*), tyrosine kinase-B (*TRKB*), and other genes such as GABA-transporter-1 (*GAT-1*), nicotinic-acetylcholine receptors (*nAChR*s), somatostatin (*SST*), and numerous calcium binding proteins (Akbarian et al. 1995; Breese et al. 2000; Guidotti et al. 2000, 2005; Woo et al. 2005; Lewis et al. 2005; Costa et al. 2007; Hashimoto et al. 2008; Mellios et al. 2009; Mill et al. 2008) is decreased in GABAergic neurons of the *Stratum oriens* and *Straum reticularis* of the hippocampus, or the basal ganglia, or in GABAergic neuronal populations located either in the upper cortical layers or proximal to layer III/V pyramidal neurons of SZ patients (Ruzicka et al. 2007; Veldic et al. 2007). However, not all genes expressed in GABAergic neurons are downregulated in psychosis. In fact, the expression of GAD65 mRNA and protein is unchanged in GABAergic neurons of SZ and BPD patients (Guidotti et al. 2000). This suggests that the downregulation of GABAergic function is not due to a loss of GABAergic neurons.

There is also abundant evidence that glutamatergic transmission may be altered in SZ (Homayoun and Moghaddam 2007; Lisman et al. 2008). Recent data support the concept that insufficient stimulation of NMDA-selective glutamate receptors (GRIN1/GRIN2A receptor containing assemblies) expressed by GABAergic interneurons leads to insufficient GABA release at synapses on cortical pyramidal neurons (Belforte et al. 2010). This could explain why NMDA receptor antagonists such as phencyclidine and dizocilpine (MK-801) induce psychotic episodes when these compounds are ingested (Lisman et al. 2008). It also explains, in part, why metabotropic glutamate receptor (mGluR) agonists that facilitate glutamate release onto GABAergic interneurons may prove beneficial to SZP (Conn et al. 2008). Because most of the observed cortical mRNA changes in SZP have been localized to GABAergic interneurons (at least in the cortex), our

hypothesis is consistent with a disruption of pyramidal neuron synchronization mediated by an inhibitory hypofunction occurring at the level of presynaptic interneurons.

This model was also suggested by recent molecular and behavioral findings in which the NR1 subunit gene (*Grin1*) of the NMDA-selective glutamate receptor complex was selectively knocked out in cortical and hippocampal GABA interneurons of mice (Belforte et al. 2009). Using the cre-loxP system, these investigators created conditional mutant mice in which the NR1 subunit of the NMDA-selective glutamate receptor was ablated during early postnatal development. Interestingly, the loss of the NR1 subunit in ~50% of cortical and hippocampal inhibitory interneurons resulted in the onset of SZ-related symptoms in adolescence. This included novelty-induced hyperlocomotion, mating and nest-building defects, anhedonia, and anxiety-like behaviors. In addition, social memory, spatial working memory, and prepulse inhibition were also impaired. Ultimately, these mice showed reduced expression of Gad67 and parvalbumin accompanied by disinhibition of cortical pyramidal neurons and reduced neuronal synchronization (Belforte et al. 2010). These data are consistent with the hypothesis that reduced glutamatergic stimulation of cortical GABA interneurons results in decreased GABA release and hence inhibitory hypofunction.

This hypothesis (Fig. 2.1) also accounts for both the GABA neuron deficits and the glutamatergic deficits that have thus far been described in SZ and BPD (Lisman et al. 2008). We suggest that NMDA-selective glutamate receptors on cortical

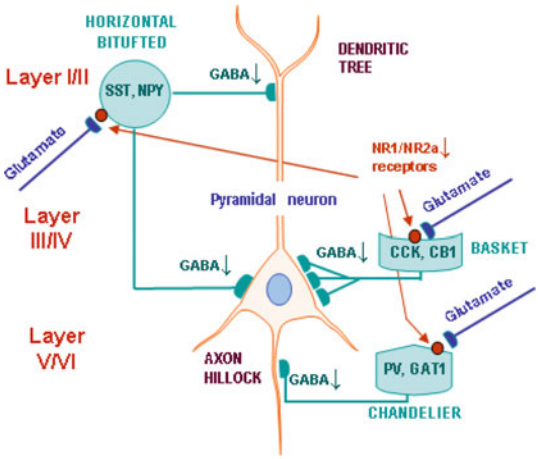


**Fig. 2.1** Cortical circuitry links multiple neuronal subtypes. Phenotypically distinct neurons reside in different cortical layers. GABAergic interneurons (aqua) function to modulate the output of pyramidal (tan) and other neurons. We propose that the reduced expression of GAD1 (also known as GAD67) and other GABAergic markers (SST, NPY, CCK, CB1, PV, and GAT1), along with reduced levels of GRIN1- and GRIN2A-containing glutamate receptors, contributes to reduced GABA release. This GABA hypofunction causes decreased pyramidal neuron synchronization

GABA neurons are reduced in SZ due to an epigenetic defect in these GABA neurons arising from upregulation of DNA methyltransferase 1 gene (*DNMT1*). As described below, the hyperexpression of DNMT1 results in promoter methylation and mRNA downregulation of selected genes expressed in GABA neurons of affected individuals.

## 2.3 Downregulation of *GAD67* and Reelin Promoters in SZ and BPD

GAD67 is the most important enzyme regulating GABA synthesis and function (Soghomonian and Martin 1998; Guidotti et al. 2005). Consequently, it has been hypothesized that the reduced expression of GAD67 in telencephalic GABAergic neurons of psychotic patients markedly reduces the effectiveness of the GABAergic inhibitory neurotransmission that impinges on the dendrites and on the initial axon segments of pyramidal neurons (Fig. 2.1). This deficit of inhibitory neurotransmitters disrupts the intermittent synchronization of pyramidal neuron firing that is critical to the most advantageous performance of cognitive function (Gonzalez-Burgos and Lewis 2008).

In psychosis, not only GAD67 but also reelin is markedly downregulated (Impagnatiello et al. 1998; Fatemi et al. 2000; Guidotti et al. 2000; Ruzicka et al. 2007; Veldic et al. 2007). Reelin is a large (400 kDa) extracellular matrix protein synthesized in large quantities in hippocampal GABAergic interneurons and in GABAergic neurons of cortical layers I and II. Upon secretion from GABAergic interneurons, reelin adheres to postsynaptic densities located on dendritic spines and shafts of pyramidal neurons (D'Arcangelo et al. 1995; Costa et al. 2001). As a result, reelin is believed to play a significant role not only in dendritic spine formation and maturation but also in glutamate receptor homeostasis (Costa et al. 2001; Levenson et al. 2008). On the basis of this role, reduced GAD67 and reelin signaling is almost certainly a reason for the reduced number of dendritic spines (Costa et al. 2001), decreased numbers of glutamate receptors (Levenson et al. 2008), increases in muscimol binding (Benes et al. 1992), and the compensatory increase of postsynaptic $\alpha5$ (Guidotti et al. 2005) and $\alpha2$ (Lewis et al. 2005) $GABA_A$ receptor subunits present in pyramidal neurons of prefrontal cortex (PFC) in SZ and BPD patients.

Because of this evidence, we and others (Benes et al. 1992; Akbarian et al. 1995; Guidotti et al. 2000, 2005; Lewis et al. 2005) have suggested that SZ or BPD are diseases characterized predominantly by a deficit of GABAergic function. Not only that, we have postulated that GABAergic neurotransmission may become an important primary research target in the development of more efficient and less toxic treatments for major psychosis. For instance, a positive allosteric modulator of GABA action at $GABA_A$ receptors that is devoid of tolerance liability and sedative action is, in our judgment (Guidotti et al. 2005), a significant candidate for use in therapeutic interventions in psychosis and should be further investigated.

## 2.4 Epigenetic Mechanisms Are Probably a Component in the GABAergic Dysfunction Expressed by SZ and BPD Patients

Since the deficit of GAD67 and reelin mRNA expression in GABAergic neurons of psychotic patients can be ascribed to genetic abnormalities (copy number variations, deletions, or polymorphisms) in only a few cases, the inheritance and selective pathology of GABAergic neurons may be better understood in the context of epigenetic factors acting on susceptible candidate genes (Costa et al. 2007; Mill et al. 2008; Ptak and Petronis 2008). This hypothesis is supported by data from SZ and BPD patients that show (1) an increased number of cortical GABAergic neurons that stain positive for DNMT1 and DNMT3a mRNA and protein (Veldic et al. 2007; Zhubi et al. 2009); (2) an increased PFC level of *S*-adenosyl-methionine (SAM), the universal methyl donor utilized by DNA-methyltransferases (DNMTs) (Guidotti et al. 2007); and (3) the promoter hypermethylation in reelin and other genes observed in GABAergic neurons (Chen et al. 2002; Abdolmaleky et al. 2005; Grayson et al. 2005; Mill et al. 2008).

## 2.5 Upregulation of DNA-Methyltransferases and Downregulation of GABAergic Promoters

In our laboratory, studies of postmortem brain have shown that the downregulation of GAD67 and reelin mRNAs in the PFC and basal ganglia of psychotic patients is associated with a significant increase of DNMT1 and DNMT3a in GABAergic neurons (Veldic et al. 2004, 2005, 2007; Ruzicka et al. 2007; Zhubi et al. 2009). DNMTs are the enzymes that transfer a methyl group from SAM to cytosines, most often in a CpG dinucleotide context (Van Emburgh and Robertson 2008). It is notable that in the neocortex, DNMT1 and DNMT3a, which are the most abundant DNA methyltransferases present in the brain, are highly expressed in GABAergic interneurons and are expressed at very low levels in pyramidal neurons and in glial cells (Veldic et al. 2004, 2005, 2007; Ruzicka et al. 2007; Zhubi et al. 2009).

In primary cultures of mouse cortical GABAergic neurons, we showed that an antisense-mediated reduction of Dnmt1 protein was accompanied by a reduction of *Reln* and *Gad67* promoter methylation and by increased reelin and *Gad67* mRNA expression. These data support the importance of Dnmts as an epigenetic regulator of gene expression in GABAergic neurons (Noh et al. 2005). More significantly, an antisense-induced knockdown of *Dnmt1* blocks the methionine-induced downregulation of reelin and *Gad67* mRNA expression (Noh et al. 2005). We recently reported (Satta et al. 2008) that the decrease (~50%) of cortical *Dnmt1* mRNA and protein induced in mice following protracted subcutaneous nicotine administration (0.75–2.5 mg/kg, four times a day for 4 days) is associated with a comparable

decrease of Dnmt1 protein, a 40–50% decrease of $Gad_{67}$ promoter methylation, and an overexpression of Gad67 protein in GABAergic interneurons. The increase of DNMT1 and to a lesser extent of DNMT3a mRNA in the brains of SZ and BPD patients is probably specific to these illnesses. In contrast, in the brains of suicidal/depressed patients, an increase of DNMT3b but not DNMT1 or DNMT3a mRNA has been reported (Poulter et al. 2008). It appears that the overexpression of DNMT1 and DNMT3a mRNA in the brains of SZ and BPD patients cannot be ascribed to demographic factors (gender, onset of illness, or duration of illness) or to the type, dose, or duration of antipsychotic administration. In fact, there was no change in cortical Dnmt1 mRNA content in mice that received haloperidol (1 mg/kg) or clozapine (5 mg/kg) subcutaneously for 21 days (Satta, this laboratory, personal communication). These data are in keeping with the concept that the downregulation in the expression of various genes in GABAergic neurons of SZ and BPD patients may be related to a DNMT1/DNMT3a-mediated methylation of the corresponding promoters (Costa et al. 2007; Mill et al. 2008).

This conclusion may be related to clinical reports dating to the late 1960s indicating that daily administration of high doses of methionine exacerbated or even triggered psychotic episodes in a SZ patient subpopulation (Wyatt et al. 1971). Since the administration of high doses of methionine increases the brain content of SAM (Tremolizzo et al. 2002, 2005), it may be inferred that the increased cortical availability of SAM associated with the increased expression of *DNMT*s may be responsible for this hypermethylation of promoters in SZ and BPD patients (Guidotti et al. 2007).

Taken together, these data suggest the hypothesis that the GABAergic mRNA downregulation occurring in SZ and BPD is likely the consequence of transcriptional repression due to promoter hypermethylation directly induced by DNMT overexpression. An alternative hypothesis is that the inhibitory action of DNMTs on GABAergic gene expression could be mediated by an interaction of DNMT with specific chromatin repressor proteins (e.g., methyl-CpG binding domain proteins, SIN3A, and histone deacetylases [HDACs]), which repress transcription via a modification of chromatin structure (i.e., shifting chromatin from a permissive open conformation to a repressive closed conformation).

Previous studies from our laboratory have shown that in the PFC, reelin expression in humans is inversely related to *DNMT1* expression (Veldic et al. 2004, 2005, 2007; Ruzicka et al. 2007; Zhubi et al. 2009) and in human neuroprogenitor (NT2) cells is regulated through changes in methylation status and chromatin structure in the vicinity of the promoter (Kundakovic et al. 2009). A goal of our recent research has been to establish the precise molecular mechanism(s) responsible for the DNMT-mediated epigenetic regulation of genes encoding reelin and GAD67. Here, we report our findings from in vitro studies that (1) confirm the coordinated regulation of the human reelin and *GAD67* promoters and (2) elucidate the molecular mechanisms that underlie this regulation.

An appreciation of this information provides a mechanistic rationale for our epigenetic hypothesis of SZ and BPD and for developing new pharmacological treatments for these diseases.

## 2.6 Epigenetic Drugs Coordinately Upregulate Reelin and *GAD67* mRNA Expression

We first tested the hypothesis that human reelin and GAD67 genes are coordinately regulated through epigenetic mechanisms. We treated NT2 cells with two different classes of epigenetic drugs, DNMT inhibitors and HDAC inhibitors. Our studies demonstrated that three distinct DNMT inhibitors: a DNA intercalator doxorubicin (DOXO) and two nucleoside analogues, 5-aza-2′-deoxycytidine (AZA) and zebularine (ZEB), are able to significantly increase reelin and GAD67 mRNA levels (Kundakovic et al. 2007, 2009). Similarly, three structurally unrelated HDAC inhibitors: MS-275 (benzamide derivative), valproic acid (VPA; aliphatic acid), and trichostatin A (TSA; hydroxamate) induce reelin and GAD67 mRNA expression in our cell system (Kundakovic et al. 2009).

To strengthen the conclusion that reelin and *GAD67* promoters are coordinately regulated, we further examined the expression patterns of these mRNAs following treatment with the DNMT inhibitor DOXO and the HDAC inhibitor MS-275 (Figs. 2.2 and 2.3). Importantly, the detailed studies with DOXO and MS-275 produced similar findings and demonstrated that reelin and GAD67 mRNA induction occurs (a) in a comparable dose-dependent manner (as shown by the very

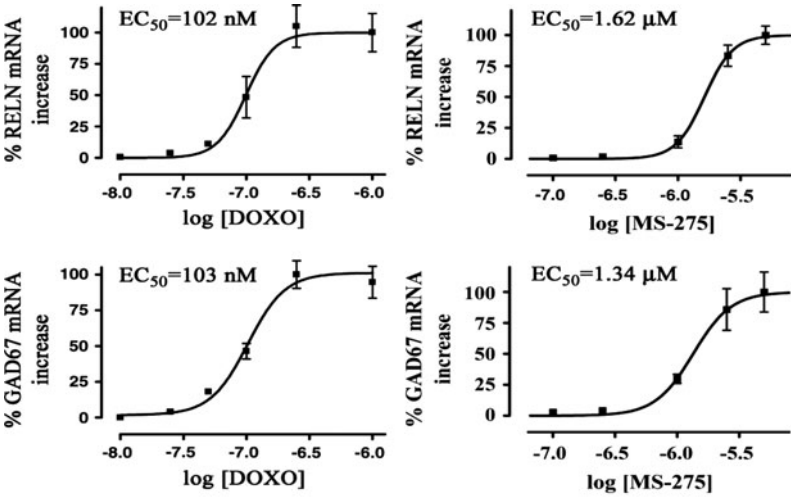


**Fig. 2.2** DOXO and MS-275 lead to dose-dependent increases in reelin and GAD67 mRNAs. Results of quantitative analysis of reelin (RELN) and GAD67 mRNA levels following 48-h treatments of NT-2 cells with (**a**) DOXO (0.01–1 μM) and (**b**) MS-275 (0.1–10 μM) are presented as dose–response curves. RT-PCR data are plotted as a percent of maximal (reelin or GAD67) mRNA increase (*y*-axis) as a function of log [drug concentration] (shown on the *x*-axis). $EC_{50}$ is the concentration of drug that leads to 50% of maximal reelin or GAD67 mRNA increases. Results are expressed as the mean ± S.E.M. of three independent experiments. Dose–response curves are generated using Prism version 5 (GraphPad Software, San Diego, CA), and $EC_{50}$ values are compared by *F*-test. Panels **a** (Kundakovic 2009) and **b** (Kundakovic et al. 2007) are reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics

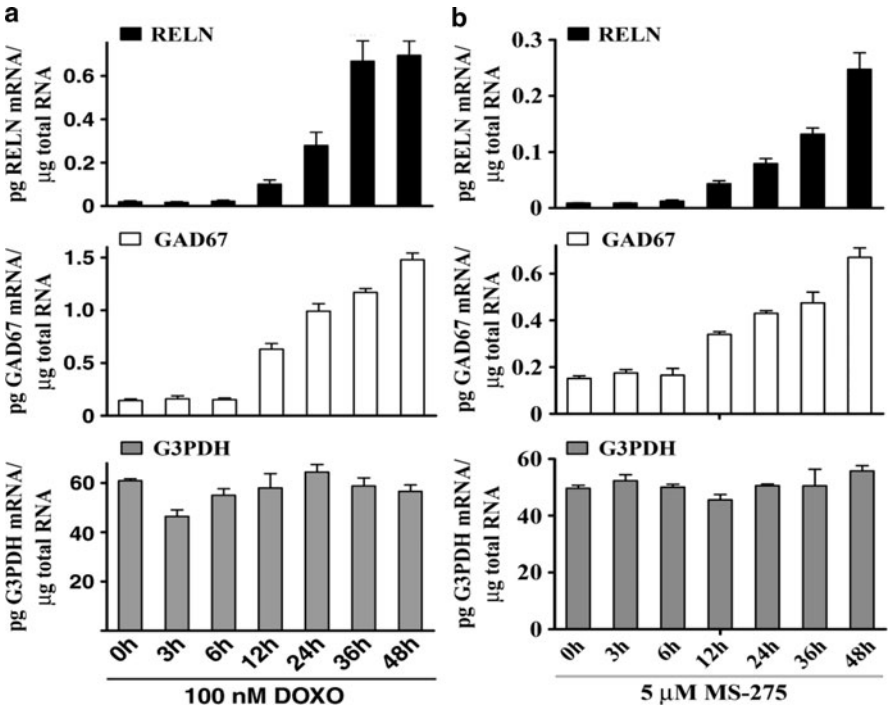


**Fig. 2.3** Both DOXO and MS-275 increase reelin and GAD67 mRNA expression in a similar time-dependent manner. Results of quantitative analysis of reelin (RELN), GAD67, and G3PDH mRNA levels in NT-2 cells treated for various times with (**a**) 100 nM DOXO or (**b**) 5 µM MS-275. Data (mean ± S.E.M. from at least three independent experiments) are presented as amounts (pg) of reelin, GAD67, and G3PDH mRNA per 1 µg of total RNA at the indicated time points (*x*-axis). Panels **a** (Kundakovic 2009) and **b** (Kundakovic et al. 2007) are reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics

similar $EC_{50}$ and $EC_{100}$ values for the induction of both mRNAs for each drug) (Fig. 2.2); and (b) within the same time frame (both mRNAs begin to be induced ~12 h after starting individual DOXO or MS-275 treatments) (Fig. 2.3). The similar concentration-dependence and temporal activation patterns of reelin and GAD67 mRNAs by drugs that target the epigenome strongly support our hypothesis that these two genes are coordinately regulated through epigenetic mechanisms.

## 2.7 Activation of the Reelin and *GAD67* Promoters Is Associated with Downregulation of the DNMT1, DNMT3A, and DNMT3B Proteins

It is of interest that both groups of drugs (DNMT and HDAC inhibitors) downregulate DNMT1 protein, suggesting a link between DNMT1 content and reelin gene and GAD67 mRNA expression (Figs. 2.4a, b). Interestingly, the DNMT

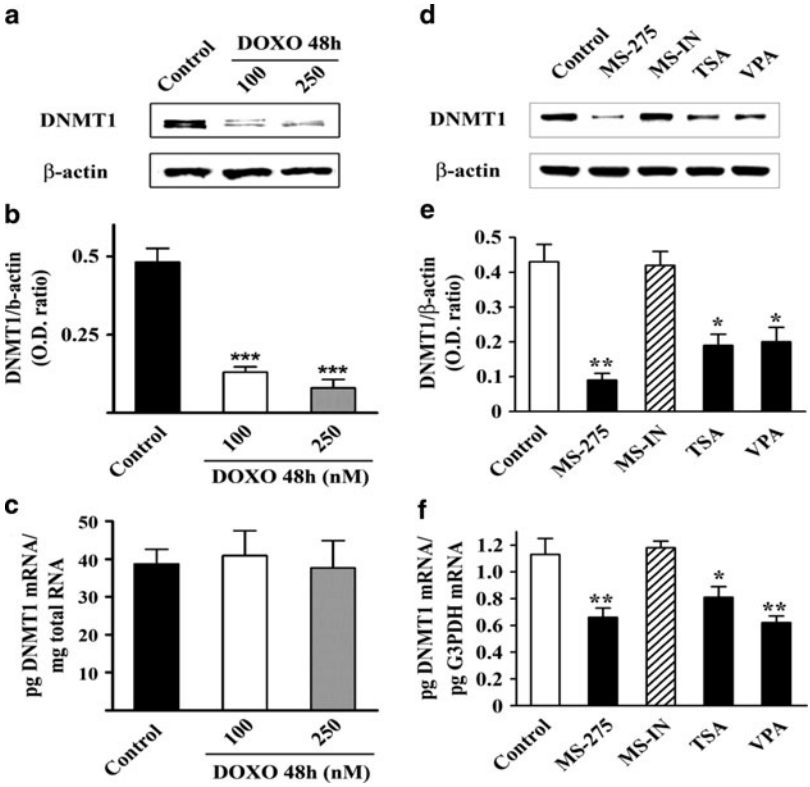


**Fig. 2.4** DNMT inhibitors and HDAC inhibitors downregulate DNMT1 protein expression. The representative Western immunoblots and the ratio of the DNMT1 band over the area of the β-actin band in nuclear extract protein samples from untreated cells (control) and cells treated with either (**a** and **b**) DNMT inhibitor DOXO (100 and 250 nM, 48 h) or (**d** and **e**) HDAC inhibitors MS-275 (5 μM, 48 h), TSA (0.3 μM, 24 h), VPA (5 mM, 24 h), and an inactive stereoisomer of MS-275 (MS-IN, 5 μM, 48 h); *bar graphs* show DNMT1 mRNA levels in the same DOXO-treated (**c**) and HDAC inhibitor-treated cells (**f**) obtained using a competitive RT-PCR assay. Data are presented as amount of DNMT1 mRNA (in pg) per 1 μg total RNA and normalized to the corresponding G3PDH mRNA levels. Data represent the mean ± SEM. ***$p < 0.001$; *$p < 0.05$ vs. control group. (One-way ANOVA followed by Bonferroni test.) Panels **b**, **c** (Kundakovic 2009) and **d**, **e** (Kundakovic et al. 2007) are reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics

inhibitor DOXO does not change DNMT1 mRNA levels (Fig. 2.4c), implying that DNMT inhibitors reduce DNMT1 posttranscriptionally. On the other hand, HDAC inhibitors decrease DNMT1 mRNA expression under the same conditions (Fig. 2.4d–f), suggesting that these drugs downregulate *DNMT1*, at least in part, by affecting its mRNA synthesis and/or degradation. Importantly, the downregulation of DNMT1 precedes reelin and *GAD67* mRNA induction (Kundakovic et al. 2009), implicating a role for this protein in regulating reelin and *GAD67* promoters. Importantly, the activation of reelin and *GAD67* mRNA expression with both

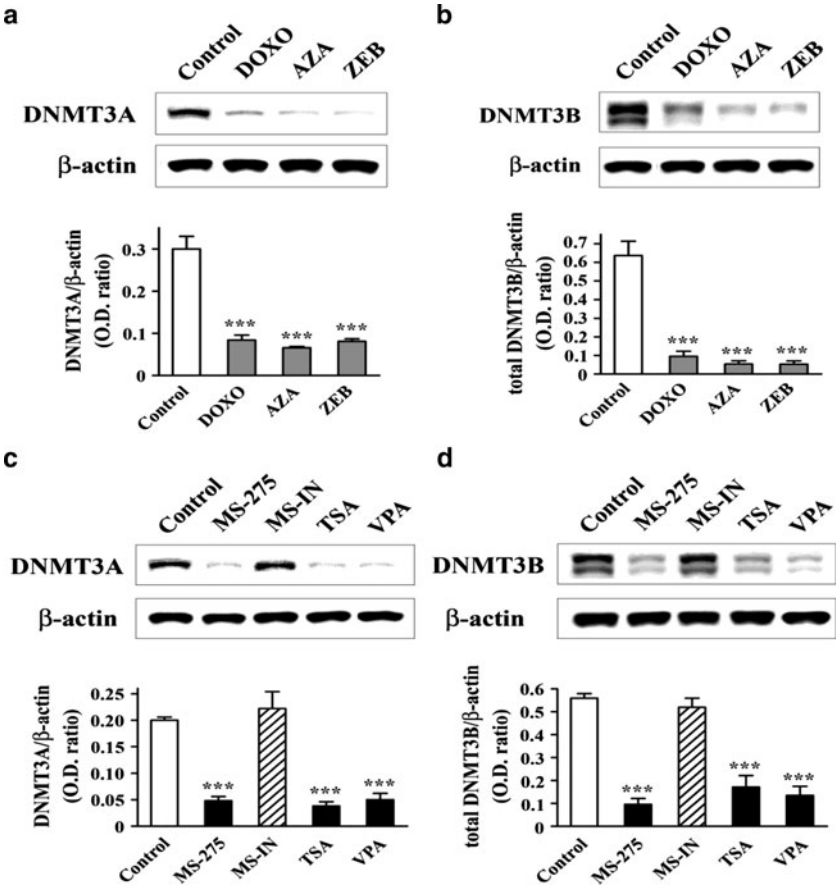


**Fig. 2.5** DNMT and HDAC inhibitors decrease DNMT3A and DNMT3B protein levels. Western blot analyses of DNMT3A and DNMT3B proteins were performed using nuclear extracts prepared from control cells and cells treated with either DNMT inhibitors (**a** and **b**) or HDAC inhibitors (**c** and **d**). For assays with DNMT inhibitors, cells were treated with 250 nM DOXO (48 h), 5 μM AZA (48 h), or 500 μM ZEB (48 h followed by 48-h incubation with untreated medium). HDAC inhibitor treatments were performed as follows: 5 μM MS-275 (48 h), 5 μM MS-IN (48 h), 0.3 μM TSA (24 h), and 5 mM VPA (24 h). In all cases (**a**–**d**), representative Western blots are shown (*upper panels*) together with the graphs depicting the ratio of the DNMT3A or DNMT3B (both isoforms) band over the area of the β-actin band (*lower panels*). Data represent the mean ± S.E.M. of three independent experiments. ***$p < 0.001$ vs. control group (one-way ANOVA followed by Bonferroni test). Reprinted (Kundakovic et al. 2007) with permission of the American Society for Pharmacology and Experimental Therapeutics

DNMT and HDAC inhibitors was also associated with the downregulation of two other DNMT enzymes, DNMT3A and DNMT3B, in nuclei of NT-2 cells (Fig. 2.5). Figure 2.5a shows the effects of DNMT inhibitors on DNMT3A protein levels, while Fig. 2.5c shows the same for various HDAC inhibitors. Similarly, Fig. 2.5b

shows the results obtained using selected DNMT inhibitors on DNMT3B protein with comparable results using various HDAC inhibitors shown below (Fig. 2.5d). The inactive enantiomer of MS-275 had no effect on the level of either protein.

## 2.8 Epigenetic Drugs Facilitate the Dissociation of DNMT-Containing Repressor Complexes from Reelin and *GAD67* Promoters

Our studies provide evidence that all three DNMT proteins, DNMT1, DNMT3A, and DNMT3B, might participate in the formation of transcriptional repressor complexes at the *reelin* and *GAD67* promoters (Fig. 2.6). These complexes also include MeCP2 and HDAC1 proteins. Our data support the concept that treatment with DNMT inhibitors (Kundakovic et al. 2009) and HDAC inhibitors (Fig. 2.6) results in the dissociation of all three DNMT proteins, together with MeCP2 and HDAC1, from both promoters. Increased local histone acetylation was also observed, implying that both classes of drugs facilitate the relaxation of chromatin

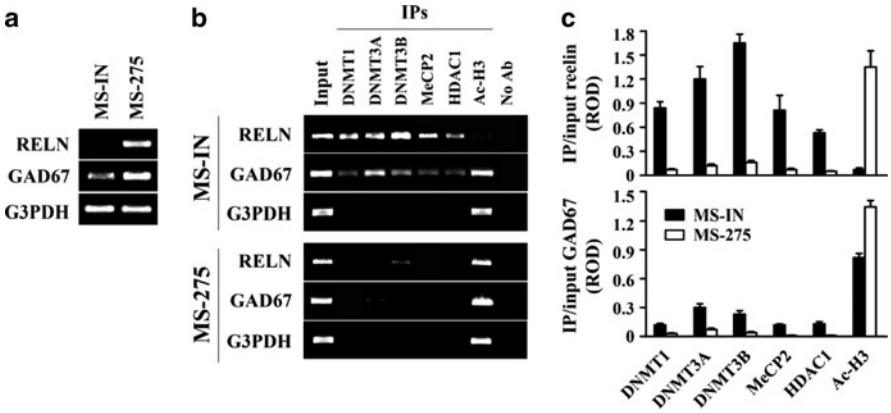


**Fig. 2.6** Reelin and GAD67 gene activation is accompanied by dissociation of repressor complexes from the corresponding promoters. NT-2 cells were treated with either 5 μM of HDAC inhibitor MS-275 or 5 μM of its inactive stereoisomer MS-IN for 48 h. (**a**) RT-PCR analysis confirmed that reelin and GAD67 mRNAs were induced by MS-275 compared to MS-IN treatment. (**b**) The corresponding chromatin preparations were immunoprecipitated with DNMT1, DNMT3A, DNMT3B, MeCP2, HDAC1, and Ac-H3 antibodies (IPs). Nonimmunoprecipitated samples were used as negative controls (No Ab). DNA isolated from inputs, Ips, and control samples were PCR-amplified using primers specific for the reelin, GAD67, and G3PDH promoter regions. Relative optical densities (RODs) of the bands derived from ethidium bromide-stained gels were quantified. (**c**) Graphs show the results (mean ± S.E.M. of three independent experiments) of semiquantitative analysis of the occupancy of DNMT1, DNMT3A, DNMT3B, MeCP2, HDAC1, and Ac-H3 on the reelin (*upper panel*) and GAD67 (*lower panel*) promoters in MS-IN- and MS-275-treated cells, normalized to input DNA (each comparison MS-IN vs. MS-275 showed a statistical significance of at least $p < 0.05$, *t*-test). Reprinted (Kundakovic et al. 2007) with permission of the American Society for Pharmacology and Experimental Therapeutics

surrounding the *reelin* and *GAD67* promoters. In addition, each inhibitor reduces total nuclear DNMT enzyme activity and facilitates a reduction in DNA methylation in the same *reelin* and *GAD67* promoter regions that are associated with changes in chromatin structure (Kundakovic et al. 2009). These data imply that the formation of the repressor complexes is likely DNA-methylation dependent. Furthermore, we suggest that promoter demethylation might not be required for a slight to moderate induction of *reelin* and *GAD67* transcription, but is likely relevant for maximal activation of these two promoters.

## 2.9 Discussion

Results from our studies provide evidence that the human *reelin* and *GAD67* promoters are coordinately regulated through epigenetic mechanisms and also suggest an underlying molecular mechanism to understand this regulation. Our data imply that both promoters are negatively regulated through methylation-dependent recruitment of transcriptional repressor complexes containing DNMT1, DNMT3A, DNMT3B, MeCP2, and HDAC1 proteins. These complexes reduce the transcriptional activity of the promoters by shifting the surrounding chromatin into a more compact state, thus resulting in decreased transcription factor accessibility (Fig. 2.7). While our data are directly applicable to the epigenetic regulation of the *reelin* and *GAD67* promoters in neuronal progenitor cells, an increasing body of evidence suggests that similar regulatory mechanisms are operative in adult GABAergic neurons (Costa et al. 2004, 2006; Szyf et al. 2008).

Further, this study gives new insight into the molecular mechanisms that underlie the downregulation of reelin and *GAD67* mRNAs in the brains of SZ. It has been reported that the reductions in reelin and GAD67 transcripts correlate with increased DNMT1 and HDAC1 expression in the same GABAergic neurons (Hayes 1989; Ruzicka et al. 2007; Benes et al. 2007). Therefore, we propose that the upregulation of DNMT1 mRNA could promote downregulation of the *reelin* and *GAD67* genes by inducing promoter hypermethylation (Abdolmaleky et al. 2005; Chen et al. 2002; Grayson et al. 2005) and increased binding of DNMT1- and HDAC1-containing corepressor complexes to the reelin and *GAD67* promoters. According to our data, these complexes most likely also contain additional DNMTs (DNMT3A and DNMT3B) and MeCP2 and other proteins as well. However, further studies with postmortem human brains will be necessary to confirm this hypothesis.

Additionally, we would like to suggest a new approach for the treatment of SZ that focuses on the reactivation of mRNA expression profiles that are downregulated due to modifications in the epigenome. We report that treatment of neuronal progenitor cells with various DNMT and HDAC inhibitors leads to a robust induction of reelin and GAD67 mRNAs. Furthermore, we demonstrate that both classes of epigenetic drugs target DNMT1 and HDAC1, which are aberrantly expressed in SZ (Fig. 2.7). These drugs downregulate DNMT1 and directly or indirectly inhibit the repressor activity of HDAC1. Moreover, the same drugs induce

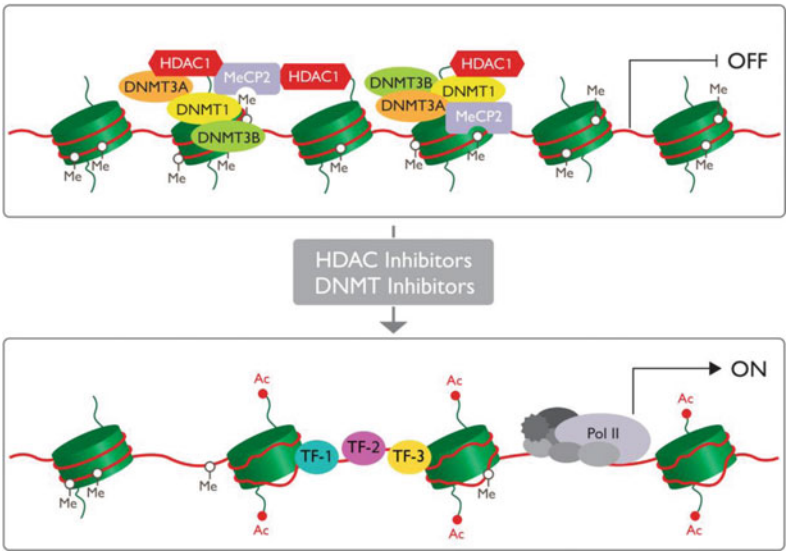


**Fig. 2.7** Hypothetical model for promoter activation by HDAC and DNMT inhibitors. For simplicity, promoters are shown as either silent (repressed – OFF) or fully active (ON). The transcriptionally inactive chromatin structure surrounding the indicated promoter region (*upper panel*) is the consequence of cytosine methylation and subsequent recruitment of repressor proteins, including DNMT1, DNMT3A, DNMT3B, MeCP2, and HDAC1 (most likely others, also). The downregulation of DNMT proteins (by DNMT inhibitors and HDAC inhibitors), together with the inhibition of HDAC enzymatic activity and the decrease in MeCP2 expression (in the case of the HDAC inhibitors), results in dissociation of these repressor complexes. This leads to DNA demethylation, histone acetylation, and a relaxation of the chromatin surrounding the respective regulatory regions (*lower panel*). The more open chromatin configuration allows the recruitment of specific transcription factors (TFs), such as Sp1 and the general transcriptional machinery (*gray shapes*) to the promoters. Collectively, this results in the drug-induced epigenetic changes leading to promoter activation (as we have proposed for the reelin and GAD67 genes)

changes in the methylation status of the CpG-island-containing reelin promoter that is hypermethylated in the brains of SZ patients (Veldic et al. 2004, Abdolmaleky et al. 2005, Grayson et al. 2005). Therefore, these data provide a mechanistic rationale for our hypothesis that HDAC inhibitors and DNMT inhibitors used either individually or in combination may represent a novel pharmacological approach for correcting reelin and GAD67 mRNA levels, and the GABAergic deficits associated with SZ (Guidotti et al. 2005; Levenson 2007).

A recent study examined alterations in GABA-related mRNAs in the PFC of subjects with SZ (Hashimoto et al. 2008). Reduced mRNA levels corresponding to presynaptic regulators of GABA function, numerous neuropeptides, and GABA-A receptor subunits were detected. In addition, studies show that the downregulation of NMDA receptors in GABA neurons could be the consequence of a primary epigenetic defect (Belforte et al. 2010). These data are consistent with the GABA deficit hypothesis proposed by us (Guidotti et al. 2005) and others (Lewis et al. 2005;

Benes et al. 2007). Additional work will identify which mRNAs are activated by various HDACs, in which brain regions and in which neurons. As the collection of these compounds increases, it should not be too long before researchers are able to design these drugs to target subclasses of HDACs showing selective expression patterns in the desired brain regions (Broide et al. 2007).

Preliminary data in rodents support the notion that MS-275 crosses the blood–brain barrier and increases histone acetylation in the frontal cortex of treated animals (Simonini et al. 2006). However, a recent pharmacokinetic study using positron emission tomography (PET) showed that MS-275 exhibits poor brain penetration (Hooker et al. 2010). So while the levels of MS-275 that cross into the brain may be sufficient for some assays, this issue needs additional clarification in terms of new drug design. Increasing attention is being paid to various HDAC inhibitors in the context of therapeutic intervention for certain cancers. It seems prudent that a similar approach may prove beneficial in reactivating genes that are downregulated as a consequence of promoter hypermethylation, as we have suggested occurs in SZ (Grayson et al. 2010). For example, in addition to *reelin* and *GAD67*, the promoter corresponding to the NMDA receptor subunit 2A is embedded in a CpG island and is downregulated in the postmortem cortex of SZ subjects (Woo et al. 2005, 2008). Interestingly, this reduced expression is in parvalbumin-expressing neurons of the cortex that also contain GAD67 and reelin (Noh et al. 2005). Clearly, the glutamatergic input onto cortical GABA interneurons plays a key role in determining synaptic GABA release onto efferent pyramidal neurons. This suggests that the two major conceptual schools that characterize the dysfunctional neural circuits that define SZ may contribute to an integrated hypothesis. That is, glutamatergic hypofunction and GABAergic hypofunction are linked provided they function at the level of the same cortical GABA neurons. Thus, it is possible that drugs acting at the level of chromatin remodeling could induce each of the mRNAs downregulated in SZ, thereby correcting the GABA neuron deficits. As such, by increasing the GABA interneuron content of GAD67, reelin, and NMDA receptor subunits, relevant neurons may more appropriately respond to uncompromised afferent NMDA receptor stimulation by releasing more GABA.

**Acknowledgments** We would like to dedicate this work to the memory of our friend and colleague, Dr. Erminio Costa, University of Illinois at Chicago, who died November 28, 2009.

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# Chapter 3
# Possible Roles of DNA Methylation in Bipolar Disorder

**Tadafumi Kato**

**Abstract** Although the role of DNA sequence-based factors is implicated in bipolar disorder (BPD), traditional genetic approaches have not been very productive in the identification of causative genes. As a result, other approaches, such as epigenetics, should also be considered in the search for the molecular basis of BPD. Genomic imprinting has been suggested to play a role in disease development, as the risk of BPD acquisition in the offspring partially depends on the sex of the disease-transmitting parent. In addition, higher parental age is associated with an increased risk of BPD in the offspring, which suggests that age-dependent alteration of DNA methylation might be involved in the etiopathogenesis of the condition. Molecular effects of drugs also support the role of epigenetics; valproate, a histone deacetylase inhibitor, is effective in the treatment of mania. Consistent with the epigenetic theory of psychiatric disease, studies of monozygotic twins discordant for BPD revealed the DNA methylation differences between siblings. Additional studies support that DNA methylation patterns at certain genes are altered in the brain tissues of bipolar disease patients. Collectively, these findings suggest that the epigenetic studies may improve our understanding of the etiology and pathogenesis of BPD.

**Keywords** Bipolar disorder · Epigenetics · Monozygotic twins

## 3.1 Introduction

Bipolar disorder (BPD) is characterized by recurrent episodes of mania and depression, affecting around 1% of the population and causing severe psychosocial dysfunction. Mounting evidence, especially from twin studies, suggests that genetic

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_3,
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factors contribute to this disorder. Classical studies that focused on the identification of DNA sequence-based factors have yielded inconsistent results. More recently, genome-wide association studies were performed in a large number of BPD patients and controls, and several promising candidate genes such as *ANK3* (ankyrin G) and *CACNA1C* (L-type voltage-dependent calcium channel, alpha 1C subunit) (Ferreira et al. 2008) and single nucleotide polymorphism (SNPs) at 3p21.1 were suggested (McMahon et al. 2010); however, the odds ratios of these SNPs were less than 1.5, suggesting only a modest contribution to the etiology of BPD. In addition to common DNA polymorphisms, the role of rare variations is gaining attention, for example, rare variants of *ABCA13* [ATP-binding cassette (ABC) transporter] have been suggested to confer a risk of BPD and schizophrenia (Knight et al. 2009). Progress in next generation sequencing will soon enable whole genome – or at least the exome – sequencing in a large group of patients and controls. Such innovation will accelerate the search for rare mutations related to the disease, but it is not known such studies will fully explain the molecular basis of BPD. Epigenetic studies may shed a new light on the origin of BPD and other psychiatric diseases and significantly advance our understanding of the molecular basis of BPD, schizophrenia, autism, among numerous other brain diseases. Petronis (2003) has suggested that epigenetics may play a role in BPD based on the non-Mendelian features of this disease, such as discordance in monozygotic (MZ) twins, the critical age for susceptibility to the disease, clinical differences in males and females, and major fluctuations of the disease course. A number of epigenetic mechanisms may be involved in BPD, including partial epigenetic instability in somatic cells and epigenetic divergence in monozygotic twins, parental origin-dependent epigenetic regulation (genomic imprinting), skewed X-chromosome inactivation, inherited and acquired epigenetic misregulation of genes expressed in the brain, and several others. Some of the epigenetic studies in BPD are discussed below, and a summary is provided in Table 3.1.

## 3.2 Epigenetic Studies of BPD

### *3.2.1 Monozygotic Twins*

Kuratomi et al. (2008) searched for DNA methylation differences between two MZ twins discordant for BPD using methylation-sensitive representational difference analysis. Bisulfite sequencing analysis confirmed that DNA methylation was altered in four genes, which became the focus of further investigation. Among the four genes, *SMS* (spermine synthase) showed epigenetic differences in female subjects in a case-control study, but the direction of the change was contrary to the finding in the twins (Kuratomi et al. 2008). The gene for peptidylprolyl isomerase E-like (*PPIEL*) showed DNA methylation differences consistent both in the twins discordant for BPD and unrelated individuals affected with BP II

**Table 3.1** Main findings of DNA methylation studies in BPD

| First author (year) | Tissue | Experimental technique | Findings |
|---|---|---|---|
| Dempster et al. (2006) | Brain (cerebellum) (15 BPD, 15 controls, 15 SZ, 15 MDD) | Bisulfite modification and pyrosequencing | No methylation difference of soluble isoform of *COMT* |
| Abdolmaleky et al. (2006) | Brain (frontal lobe) (35 BPD, 35 + 5 controls, 35 + 5 SZ) | Methylation specific PCR | *MB-COMT* is hypomethylated in BPD |
| Tamura et al. (2007) | Brain (forebrain) (35 BPD, 35 controls, 35 SZ) | Methylation-sensitive restriction enzymes and real-time PCR | Lack of correlation between the levels of DNA methylation in *RELN* and age in BPD |
| Kuratomi et al. (2008) | Lymphoblastoid cells (1 discordant MZ twin pair, 16 BPI, 7 + 14 BPII, 18 controls) | MS-RDA, bisulfite sequencing, pyrosequencing | PPIEL is differentially methylated in MZ twins discordant for BPD. PPIEL is hypomethylated in BP II disorder |
| Rosa et al. (2008) | Blood and buccal swabs (63 female MZ twin pairs) | Methylation-sensitive restriction enzymes and (nonquantitative) PCR | Discordant female BPD twins showed greater methylation differences between two X-chromosomes than concordant twin pairs |
| Mill et al. (2008) | Brain (frontal cortex) (35 BPD, 35 controls, 35 SZ). Sperm (20 BPD, 20 controls) | Epigenomic profiling by DNA microarray, bisulfite modification, pyrosequencing | DNA methylation was altered in a number of genes, but all were sex specific. No DNA methylation changes in the sperm of BPD patients |
| Bromberg (2009) | Peripheral blood leukocytes (49 BPD, 27 controls) | Cytosine-extension assay | Leukocyte global DNA methylation did not differ between BPD and controls |

*COMT* the gene for catechol-*O*-methyltransferase, *MB-COMT* the gene for membrane-bound-COMT, *MS-RDA* methylation sensitive representational difference analysis, *SZ* schizophrenia, *BPD* bipolar disorder, *BP I disorder* bipolar I disorder, *BP II disorder* bipolar II disorder, *MDD* major depressive disorder, *MZ* monozygotic twins

disorder compared with controls. In the human brain, *PPIEL* is highly expressed in the pituitary gland and the substantia nigra. In another twin study, X-chromosome inactivation patterns were investigated in DNA samples from blood and/or buccal epithelial cells in a sample of 63 female MZ twin pairs concordant or discordant for BD or schizophrenia, as well as healthy MZ controls (Rosa et al. 2008). Female twins discordant for BPD showed greater DNA methylation differences in both maternal and paternal X alleles than the concordant twin pairs, which suggests that

differential skewing of X-chromosome inactivation may contribute to BPD in females and the potential involvement of X-linked loci in the disorder.

### 3.2.2 Parental Origin Effects and Genomic Imprinting

Decades ago, it was noted that father-to-son transmission of BPD is a rare event (Winokur and Tanna 1969), and initially, this was interpreted as X-linked inheritance. McMahon and colleagues (1995) provided a new interpretation for the parental origin effects in BPD, suggesting that the disease may be transmitted by genomically imprinted genes or that transmission was related to maternal effects due to mitochondrial DNA. In the case of genomic imprinting, only one of the two inherited alleles (maternal or paternal) is expressed, while the other allele is suppressed by DNA methylation and other epigenetic mechanisms. Consequently, diseases induced by mutations in imprinted genes follow a complex inheritance pattern. Although a mutant allele is transmitted from a parent, the offspring will not express the disease if the mutated gene is imprinted (Kato et al. 1996). Linkage analysis can be performed by considering the parent-of-origin of segregating DNA markers, and several earlier studies showed that BPD is linked to chromosome 18 only when the parent-of-origin effect was considered (Gershon et al. 1996). To date, the parent-of-origin effect has been utilized several times in linkage analyses of BPD; however, detection of imprinted BPD genes is a challenging task. Recent studies of parental origin-dependent differences in gene expression in the brain demonstrated that genomic imprinting is a common and far more complex phenomenon than the "on–off" type regulation of a small group of classical imprinted genes (Gregg et al. 2010). The latter study showed that most of the imprinted genes exhibit quantitative rather than qualitative differences in expression of maternal and paternal alleles. In addition, parental origin effects are highly variable across different brain regions and dependent on the developmental stage (Gregg et al. 2010).

### 3.2.3 Postmortem Brain Studies

Studies of DNA methylation in postmortem brain samples from BPD patients and controls showed differences in several candidate genes, such as *COMT* (catechol-*O*-methyltransferase) (Abdolmaleky et al. 2006, Dempster et al. 2006) and Reelin (Tamura et al. 2007), but it remains unknown if these changes are causal to BPD or if they were induced by compensatory brain mechanisms, treatment, or some other disease-related factors. Thus far, only one study performed a comprehensive microarray-based search for DNA methylation differences in the postmortem brains from BPD and schizophrenia patients ($N = 105$, brain samples from the Stanley Medical Research Foundation) (Mill et al. 2008). In this study, the enriched

unmethylated genomes were interrogated on 12K CpG island microarrays, which revealed DNA methylation differences in many genes with both brain- and nonspecific functions. Aberrant DNA methylation was detected in the genes involved in glutamatergic and GABAergic neurotransmission pathways, as well as in genes related to brain development in both BPD and schizophrenia females. Epigenetic differences were also observed at loci involved in stress response in male BPD patients. In addition to the locus- and gene-specific comparisons, the network analysis revealed a lower degree of DNA methylation modularity in the germline of male BPD patients (0.33) compared with the unaffected controls (0.47), which suggests that epigenetic dysfunction in BPD may involve a large group of genes and even whole nuclear compartments.

### *3.2.4 Miscellaneous Findings Relevant to the Epigenetic Hypothesis in BPD*

Another possible epigenetic mechanism of BPD involves abnormal DNA methylation that impairs the suppression of retroelements, such as long interspersed nuclear elements (*LINE*s) (Kan et al. 2004). *LINE*1 is a repetitive sequence in the mammalian genome with more than 500,000 copies, and this sequence comprises about 17% of the human genome. Full length *LINE*1 is about 6 kb long, containing open reading frames encoding an RNA-binding protein and a protein with endonuclease and reverse-transcriptase activities that enables *LINE* elements to transpose (Cordaux and Batzer 2009). Studying human postmortem brains, Coufal and colleagues (2009) recently reported that *LINE*1 can transpose in human neural progenitor cells, and that CpG sites at the 5′ untranslated region of *LINE*1 showed lower DNA methylation in fetal brain samples compared with skin samples. Since DNA methylation is thought to be one of the mechanisms that inactivate retrotransposons, deficiencies in *LINE*1 methylation in the brain may lead to increased de novo retrotransposition and, therefore, disruption of coding DNA sequences.

Higher paternal and maternal ages at conception were detected in the recent BPD studies (Menezes et al. 2010). Among several other interpretations, the effects of parental age may be explained by age-related epigenetic changes in the germline (Flanagan et al. 2006). Age-dependent aberrations in DNA methylation may be one of the risk factors for major psychiatric disease in the offspring (Perrin et al. 2007).

The role of DNA methylation in BPD is further supported by the fact that some drugs used to effectively treat this condition are epigenetic modifiers. Valproate, a mood stabilizer, acts in many ways to alleviate symptoms of BPD; in addition to numerous other effects, valproate inhibits class I histone deacetylases (HDAC) (Phiel et al. 2001) and alters DNA methylation through histone acetylation (Detich et al. 2003). Furthermore, *S*-adenosyl methionine (SAM), a methyl donor in the methylation of various targets, including DNA (Rydberg and Lindahl 1982), was shown to facilitate the switch from depression to elevated mood state in an open

trial of intravenous and oral SAM (Carney et al. 1989), which also argues for the involvement of epigenetic factors in mood regulation.

## 3.3 Future Directions

The study of putative epigenetic misregulation in BPD has only just begun. Some findings provide potentially important insight into the etiology of BPD, but further studies will be required to elucidate the possible role of DNA methylation. Development of microarrays specifically dedicated to DNA methylation mapping is of significant value in the whole DNA methylome studies (Feinberg 2010). Furthermore, genome-wide DNA methylation analysis at the single base-pair level is now possible using next-generation sequencing technology (Lister et al. 2009). In addition to the methylation at CpG sites, non-CpG sites have also been reported to be methylated in embryonic stem cells (Lister et al. 2009). The role of such non-CpG methylation in neural stem cells is currently unknown and requires further investigation.

Several new phenomena have recently been reported, and may prove relevant to BPD. In addition to the methylated cytosine (the fifth base pair), the sixth base pair, hydroxymethylcytosine was found to be enriched in the brain (Kriaucionis and Heintz 2009). Though the functional relevance of hydroxymethylcytosine is not known, it would be of great interest to study its role in the postmortem brain of patients affected with BPD. By considering an additional regulatory mechanism in the brain, the etiology of BPD and other diseases could potentially become more apparent.

Epigenetics is a rapidly growing, cutting-edge area of molecular biology. We hope that epigenetic strategies and approaches will help to uncover the inherited and acquired basis of BPD.

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# Chapter 4
# The Epigenetics of Depression and Suicide

**Benoit Labonté and Gustavo Turecki**

**Abstract** Major depression and suicide, which is its most severe outcome, are common problems that represent a major burden in our society. The relationship among early life adversity, depression, and suicide has already been well demonstrated, however, the molecular mechanisms mediating this relationship still remain poorly understood. From rat studies, we have recently gained major insight into some of the genomic processes that modify behavior, which result from early social environmental experiences. These processes, collectively referred to as epigenetics, are defined by chemical modifications taking place around the DNA molecule that alter the coding capacity of a gene. Accordingly, this is believed to represent an interface through which the environment can act upon our genome to modify gene expression and behavior. As such, epigenetic alterations induced by environmental factors, such as early life adversity and social stress, are found in the brain of humans and rats. In this chapter, we review the evidence in favor of epigenetic factors playing a role in depression and suicide. Animal and human postmortem studies reporting epigenetic alterations in the brain following environmental insults are discussed to generate a global scheme of epigenetic modifications, which are believed to be involved in the pathophysiology of depressive disorders and suicidality.

**Keywords** Depression · DNA methylation · Early-life adversity · Environment · Epigenetics · Histone modifications · Suicide

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*,
Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_4,
© Springer-Verlag Berlin Heidelberg 2011

## 4.1 The Burden of Depression and Suicide

Mood disorders, also known as affective disorders, are common conditions characterized by mood dysregulation and neurovegetative dysfunction (Akiskal 1995). Major depressive disorder (MDD), commonly referred to as major depression, may or may not be recurrent and represents one of the most important and common types of mood disorder. One-year prevalence estimates for MDD range between 6.4 and 10.1% (Robins and Price 1991; Regier et al. 1988; Weissman et al. 1982; Kessler et al. 2005a, b). Consequently, MDD ranks first among the most significant causes of disability and premature death, and as such, imposes a continual economic burden on society. For example, in the USA, the direct and indirect costs are estimated at $44 billion/year (Lopez and Murray 1998). The greatest loss to our society, however, is the associated mortality of MDD related to suicide. Between 50 and 70% of suicide completers die during an episode of MDD (Arsenault-Lapierre et al. 2004; Cavanagh et al. 2003), and prospective follow-up studies of MDD suggest that between 7 and 15% of these patients will die by suicide (Angst et al. 1992, 1999, 2002; Blair-West et al. 1999).

## 4.2 Early Life Adversity, Major Depression, and Suicide Risk

MDD and suicide are complex phenomena believed to result from the interaction of several factors. These include neurobiological (Mann 2003; Ernst et al. 2009a) and genetic factors (Brezo et al. 2008a), which increase individual predisposition to depression and suicide; psychological and personality traits such as impulsivity and negative affect (McGirr and Turecki 2007; Yen et al. 2009); demographic variables (Kwok and Shek 2008); and early life adversity (Brezo et al. 2008b; Fergusson et al. 2008).

Among risk factors, childhood abuse [sexual (CSA), physical (CPA)] and parental neglect are among the strongest predictors of depressive disorders (Molnar et al. 2001; Arnow 2004) and suicide (Evans et al. 2005; Santa Mina and Gallop 1998). In particular, CSA is associated with earlier age of onset of depression, chronic course, and more severe depressive outcome (Gladstone et al. 2004; Dinwiddie et al. 2000; Jaffee et al. 2002). Moreover, history of CSA increases the odds of suicidal behavior up to 12 times (Molnar et al. 2001; Bensley et al. 1999). Although less consistently, CPA and neglect in childhood are also found to modify the risk for depression onset, course, severity, and associated suicidality (Evans et al. 2005; Widom et al. 2007; Ystgaard et al. 2004). The detrimental long-term consequences of childhood adversity extend beyond MDD and suicidality. In addition to being associated with these phenotypes, childhood abuse has also been found to influence the risk of other psychiatric conditions and to predict increased comorbidity (Gladstone et al. 2004; Mullen et al. 1993, 1996; Jumper 1995). While personality traits and psychological adjustment difficulties may be

possible mediators (Johnson et al. 1999a, b; Finkelhor and Browne 1985), there is a clear major predictor effect between history of early life adversity, MDD, and suicidality, which is independent of family psychopathological background (Nelson et al. 2002).

## 4.3 Early Life Adversity and Negative Mental Health Outcomes: Molecular Mechanisms

As discussed above, the relationship between childhood adversity, major depression, and suicide is supported by substantial theoretical and empirical work. It is generally assumed that adversity during childhood impacts proper psychological development and induces maladaptive patterns of behavioral responses, which, in turn, are associated with pervasive interpersonal difficulties, enhanced reactivity to stress, and an increased risk of psychopathology. However, the molecular mechanisms that account for these relationships are poorly understood. The critical question is: "How do events occurring in childhood influence the risk of becoming depressed and dying by suicide many years later?" or alternatively stated: "What long-lasting molecular mechanisms take place as a result of the adverse life experience that could be associated with increased risk for depression and suicide?" Recently, there has been growing evidence suggesting that the genome may respond to other types of environmental stimuli, beyond those of a chemical and physical nature, through epigenetic processes. Accordingly, there is mounting evidence suggesting that our genome responds to the social environment as much as it does to the physical environment, and that the basic molecular mechanism of this response is through epigenetic modifications (see Tables 4.1 and 4.2). As such, it is possible to hypothesize that epigenetic mechanisms may account, at least in part, for the regulation of behavior as a response to environmental adversity.

Epigenetics refers to the regulation of gene expression via DNA methylation (Klose and Bird 2006), histone modifications (Kouzarides 2007), and more recently, posttranscriptional mechanisms such as microRNA (Schratt 2009a, b). Generally, it has been suggested that DNA methylation directs transcriptional repression (Klose and Bird 2006). At the chromatin level, high histone acetylation and some types of histone methylation have been associated with active transcription (Kouzarides 2007). With increasing amounts of evidence suggesting that epigenetic mechanisms may be involved in the modification of gene expression induced by environmental factors, epigenetics is now believed to be one of the molecular processes induced as a response to environmental challenges. In this sense, epigenetics may be conceptualized as the means by which our cells adapt to particular cellular and environmental conditions.

Despite the complexity and the heterogeneity of depressive disorders, common functional and physiological alterations have been consistently reported in the brain of depressed and suicide subjects. For instance, stress regulatory systems

**Table 4.1** Summary of published studies assessing epigenetic components in the animal's brain following environmental insults

| Studies | Brain regions | Genes | Findings |
|---|---|---|---|
| Weaver et al. (2004) | Hippocampus | GR | ↑ Methylation in NGFI-A binding site within GR promoter in the hippocampus of low LG/ABN pups<br>Cross-fostering reinstated normal methylation levels<br>↓ H3K9 acetylation levels in low LG/ABN pups<br>TSA treatment reinstated promoter methylation, H3K9 acetylation, NGFI-A binding and $GR1_7$ expression |
| Murgatroyd et al. (2009) | Hypothalamus [paraventricular nucleus (PVN)] | AVP | ↑ AVP expression correlated with hypomethylation at five sites within AVP enhancer following early life maternal separation in 6 weeks, 3 months, and 1 year mice<br>↓ MeCP2 binding to AVP enhancer associated with higher AVP expression in 10 days old stress mice |
| Tsankova et al. (2006) | Hippocampus | BDNF | ↓ Expression of BDNF transcripts (III and IV) following chronic social stress in mice<br>No effect of promoter methylation on BDNF expression<br>↑ H3K27 methylation in the promoter region of transcripts III and IV<br>↓ HDAC5 levels associated with ↑ H3 acetylation and ↑ H3K4 methylation in the promoter of transcripts III and IV following imipramine treatment in chronically stressed mice |
| Roth et al. (2009) | PFC | BDNF | ↓ Expression of BDNF transcripts (IV and IX) in the PFC of maternally maltreated rats<br>↑ Promoter (III and IX) methylation in the PFC of maternally maltreated rats |

[hypothalamic-pituitary–adrenal (HPA) axis, polyamines], cell signaling molecules [ribosomal RNA (rRNA)], brain-derived neurotrophic factor (BDNF), neurotrophic tyrosine kinase receptor (trkB), quaking (QKI) and neurotransmitters [gamma-aminobutyric acid (GABA)], and reelin and serotonin (5-HT) have all been suggested to be involved in depression and suicide. Indeed, altered expression of genes and proteins involved in the regulation of these systems has been reported in the brains of depressed and suicide subjects, and similar alterations have also been reported in the brains of animal models. With the growing interest in epigenetics, numerous studies suggest that these alterations could be due to epigenetic modifications. The following sections will review these findings.

**Table 4.2** Summary of published studies assessing epigenetic components in postmortem brains of suicide completers

| Studies | Brain regions | Genes | Findings |
|---|---|---|---|
| McGowan et al. (2009) | Hippocampus | GR | ↑ Methylation in NGFI-A binding site within GR promoter in the hippocampus of suicide completers with history of abuse<br>↓ Expression of GR in the hippocampus of suicide completers with history of abuse |
| McGowan et al. (2008) | Hippocampus | rRNA | Overall hypermethylation of rRNA promoter in the hippocampus of suicide completers with history of abuse<br>↓ Expression of rRNA gene in the hippocampus of suicide completers with history of abuse |
| Ernst et al. (2009b) | Frontal cortex | TrkB-T1 | ↑ Methylation in two sites within the promoter of TrkB-T1 in the frontal cortex of suicide completers<br>↓ Expression of TrkB-T1 in the frontal cortex of suicide completers |
| Ernst et al. (2009c) | Frontal cortex | TrkB-T1 | ↑ H3K27 methylation in the frontal cortex of suicide completers<br>Negative correlation between H3K27 methylation levels and TrkB-T1 expression in the frontal cortex of suicide completers |
| Poulter et al. (2008) | Frontopolar cortex (FPC)<br>Hippocampus<br>Amygdala<br>Brain stem | $GABA_A$ α1<br>DNMT1<br>DNMT3a<br>DNMT3b | *FPC*: ↓ Expression of DNMT1 mRNA in suicide completers<br>↑ Expression of DNMT3b mRNA and protein levels in suicide completers<br>↑ Methylation at two sites in the promoter region of GABAA receptor subunit α1 in suicide completers<br>*Limbic system*: ↓ Expression of DNMT1 and DNMT3b mRNA levels in suicide completers<br>*Brain stem*: ↓ Expression of DNMT3b mRNA in suicide completers |
| Grayson et al. (2005) | Occipital cortex | Reelin, GAD67 | ↑ Methylation in CREB binding sites within reelin promoter in the occipital cortex of schizophrenia subjects |

(*continued*)

**Table 4.2** (continued)

| Studies | Brain regions | Genes | Findings |
|---|---|---|---|
| Tamura et al. (2007) | Forebrain | Reelin | ↑ Methylation at three sites within reelin promoter in the forebrain of schizophrenia subjects<br>↓ Expression of reelin mRNA in the forebrain of schizophrenia subjects |
| Fiori and Turecki (2010) | PFC | SMOX, SMS | No effects of promoter's methylation in SMOX and SMS on expression levels |
| Fiori and Turecki (unpublished) | PFC | SAT1 | Negative correlation between promoter's methylation levels and expression of SAT1 |
| De Luca et al. (2009) | PFC | 5-HT2A | ↓ Methylation in the promoter region of 5-HT2A receptor associated with a C-allele (trend) in the PFC of suicide completers<br>↑ Methylation in the promoter region of 5-HT2A receptor associated with a C-allele in leukocytes of suicide attempters |
| Klempan et al. (2009b) | Frontal cortex | QKI | No difference in methylation pattern between suicide completers and controls |

## 4.4 Stress Regulatory Systems

### *4.4.1 Hypothalamic-Pituitary–Adrenal Axis*

The HPA axis is the stress regulatory system (Pariante and Lightman 2008). When facing stressors, corticotropin-releasing factor (CRF) and vasopressin (AVP) are released from the hypothalamus. CRF and AVP induce the release of adrenocorticotropic hormone (ACTH) and proopiomelanocortin (POMC) from the pituitary gland into the blood, which reach the adrenal cortex and stimulate release of glucocorticoids – cortisol in humans and corticosterone in rodents. Glucocorticoids then act at each level of the HPA axis to regulate the stress response by decreasing the release of CRF, AVP, POMC, and ACTH. The locus of regulation of the HPA axis lies in the hippocampus, where glucocorticoids bind glucocorticoid receptors (GR) and induce an inhibitory feedback on the activation of the HPA axis to return the activity of the stress response back to basal levels.

Hyperactivity of the HPA axis is a common feature in depressed patients and childhood abuse victims. High levels of salivary, plasma, and urine glucocorticoids have been reported in depressed patients (Nemeroff and Vale 2005). Similarly,

higher ACTH and cortisol levels have been reported in victims of childhood abuse following stress and dexamethasone (DEX) challenges (Heim et al. 2000, 2008). HPA axis hyperactivity is thought to be related to attenuated feedback inhibition that is normally induced by endogenous glucocorticoids at the different levels of the HPA axis. In the hippocampus, this is, in part, due to the binding of glucocorticoids to their receptor. Accordingly, rat pups raised by low licking and grooming (LG) mothers have altered the HPA axis negative feedback (Liu et al. 1997), coinciding with lower glucocorticoid receptor (GR) mRNA hippocampal levels (Liu et al. 1997), and exhibit depressive-like behavior during adulthood (Francis et al. 1999). Similarly, suicide completers with a history of childhood abuse have lower GR levels in the hippocampus (McGowan et al. 2009). Consequently, reduced levels of GR in the hippocampus of depressed patients and abuse victims could be responsible for the blunted and maladaptive responses to stress in those patients.

The GR gene is preceded by 14 noncoding exons in humans and by 10 in rats (Turner and Muller 2005; McCormick et al. 2000). These noncoding exons possess multiple transcription factor binding sites and exhibit highly variable methylation patterns (Turner et al. 2008), suggesting that they might be involved in the regulation of GR expression in different tissues. The noncoding exon $1_F$ and its rat homologue $1_7$ are specific to the hippocampus (Turner and Muller 2005) and possess a number of canonical and noncanonical binding sites for the neural growth factor inducible A (NGFI-A) transcription factor, which is expected to play a major role in GR hippocampal expression (Meaney 2001). Accordingly, it was hypothesized that promoter hypermethylation could be responsible for the down-regulation of GR in the hippocampus of rats raised by low LG/ABN mothers and in suicide completers with a history of abuse. As a matter of fact, DNA methylation in GR$1_7$ in rats and $1_F$ in human promoters was shown to be elevated in both pups raised by low LG/ABN mothers (Weaver et al. 2004) and abused suicide completers (McGowan et al. 2009). Importantly, hypermethylation was found mainly within the NFGI-A-binding sites, suggesting that it could repress the binding of this transcription factor to its cognate DNA sequence and decrease the transcription of this gene. Indeed, NGFI-A was shown to significantly increase the transcriptional activity of GR$1_F$ promoter, while artificial methylation at its binding site decreases this activity, even in the presence of NGFI-A (McGowan et al. 2009).

NGFI-A has been previously shown to recruit the histone acetylase CREB-binding protein, CBP (Weaver et al. 2007). Hypermethylation within the NGFI-A-binding site could repress both the NGFI-A binding and the recruitment of CBP to the promoter of GR. This is supported by the fact that lower H3K9 acetylation and NGFI-A levels in the GR $1_7$ promoter region were found in low LG/ABN pups (Weaver et al. 2004). Interestingly, cross-fostering pups from low LG/ABN mothers to high LG/ABN mothers during the first week of life was sufficient to reinstate basal methylation values, suggesting that these modifications can be reversed. Moreover, pharmacological challenge with the histone deacetylase inhibitor TSA raised H3K9 acetylation levels, restored methylation levels, increased NGFI-A binding in GR $1_7$ promoter, and returned GR expression to basal levels in the hippocampus. It is also noteworthy that the treated rats were less reactive to stressful conditions.

Early life stress also affects other components of the HPA axis. Recently, it has been shown that earlylife infant–maternal separation in mice induces a long-lasting increase in corticosterone secretion accompanied by increased expression of POMC and AVP in the paraventricular nucleus (PVN) of the hypothalamus. These physiological modifications were also associated with stress-coping behavioral alterations (Murgatroyd et al. 2009). The AVP gene in mice is composed of three coding exons located on chromosome 20 at locus 20p13. Four CpG islands have been identified throughout the gene, one of which is found in the intergenic region separating the neighboring tail-to-tail-oriented *Avp* and oxytocin genes, and is known to include an enhancer region in the first 2.1 kb proximal to the *Avp* gene, which is important for *Avp* expression (Gainer et al. 2001).

The regulation of *Avp* expression in stressed mice has recently been shown to involve a dual epigenetic mechanism (Murgatroyd et al. 2009). Hypomethylation at 5′ sites within the enhancer was strongly correlated with higher *Avp* mRNA expression in 6-week- to 1-year-old stressed mice compared with controls. In this sense, treatment with the demethylating agent 5-azacytidine decreased methylation levels and increased *Avp* expression in cell lines; the deletion of the enhancer almost completely abolished transcriptional activity in reporter assays. These observations are consistent with the repressive role of DNA methylation on transcriptional activity; however, no methylation difference was observed at 10 days. Interestingly, these sites were shown to be putative binding sites for the methylated CpG-binding protein, MeCP2, and, despite the repressive role of MeCP2 on transcription, increased expression of *Avp* was reported. Nevertheless, it has been previously suggested that neuronal depolarization might trigger $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) phosphorylation of MeCP2, causing its dissociation from putative targets (Chen et al. 2003; Zhou et al. 2006). In fact, higher CaMKII immunoreactivity and phosphorylated MeCP2 levels were found in *Avp*-expressing neurons in the PVN of 10-day-old stressed mice, suggesting that activity-induced phosphorylation of MeCP2 decreases its binding to the *Avp* enhancer and increases the expression of *Avp*. This effect can be mimicked by a site-specific hypomethylation in the enhancer of *Avp* induced by early life stress, which could be responsible for the long-lasting increased *Avp* expression in the PVN of those mice. These results suggest that alterations in DNA methylation found outside of the promoter might also be involved in physiological and behavioral modifications induced by environmental factors.

### *4.4.2 Polyamine System*

The polyamines are important ubiquitous aliphatic molecules known to be involved in cellular function, including growth, division, and signaling cascades (Gilad and Gilad 2003; Minguet et al. 2008); but also in stress responses, both at the cellular and behavioral level (Rhee et al. 2007; Fiori and Turecki 2008). These polyamines include putrescine, spermidine, spermine, and agmatine (Moinard et al. 2005). Moreover,

polyamine-associated molecules, including the polyamine oxidase (PAO), spermine synthase (SMS), spermidine/spermine *N*-acetyltransferases (SAT1,2), ornithine decarboxylase (ODC), and ornithine aminotransferase (OAT), are also involved in the polyamine system.

Many lines of evidence suggest that the polyamine system might be involved in MDD and suicide. For example, chronic unpredictable stress in rats decreases the expression of putrescine, spermidine, and spermine in the hippocampus (Genedani et al. 2001). Agmatine and putrescine have also been shown to induce antidepressant and anxiolytic effects in rodents through a mechanism thought to involve NMDA receptors (Zomkowski et al. 2002, 2006; Gong et al. 2006). In humans, agmatine and PAO levels were shown to be increased in the plasma and serum of depressed patients and were normalized following chronic bupropion and electroconvulsive therapy (ECT) treatment (Dahel et al. 2001; Halaris et al. 1999). Moreover, the expression of SMS, SAT1 and -2, and OAT has been shown to be altered in the limbic system of suicide completers with a history of depressive disorders (Sequeira et al. 2006, 2007). It is interesting to note that the polyamine system is also reactive to stressors (Gilad and Gilad 2003) – acute stressors have been shown to increase ODC activity and putrescine and agmatine levels in the brain of rodents, while chronic stress increases ODC activity and putrescine, spermine, and spermidine levels (Gilad and Gilad 2003; Aricioglu et al. 2003). Furthermore, the emergence of the characteristic adult polyamine stress response is correlated with the cessation of the hyporesponsive period of the HPA axis system (Gilad et al. 1998).

Given the alterations in the polyamine gene expression reported in the brains of suicide completers with a history of depression, the methylation patterns of particular polyamine genes were recently assessed. Promoter methylation was found to be negatively correlated with the expression of *SAT1* (Fiori et al. unpublished results), although not with the gene for spermine oxidase (*SMOX*) and *SMS* (Fiori and Turecki 2010). Moreover, no association was found between H3K27me3 modification in the promoter region of SMS and SMOX and suicide completion or expression of these genes in BA 8/9. These findings suggest that epigenetic alterations in the promoter region of genes involved in the polyamine synthesis do not play a major role in suicidal behavior, although they may partly explain why polyamine gene expression is decreased in the brain of suicide completers.

## 4.5 Cell Signaling

### *4.5.1 Ribosomal RNA*

The rRNA is the principal component of the ribosome. Its role is to decode the mRNA into amino acids and to provide enzymatic activity allowing the right amino acid to be added to the synthesized polypeptides. Consequently, rRNA is a bottleneck structure for protein synthesis, allowing the right proteins to be synthesized depending on the needs of the cell.

The rRNA promoter is composed of two regulatory regions, namely the upstream control element (UCE) and the core promoter that binds the upstream binding factor (UBF) (Haltiner et al. 1986; Learned et al. 1986; Ghoshal et al. 2004). The expression of rRNA genes has been shown to be epigenetically regulated both in mice (Santoro and Grummt 2001) and humans (Ghoshal et al. 2004; Brown and Szyf 2007). In mice, the recruitment of transcription repressors has been suggested to induce chromatin modifications leading to methylation of a single CpG found within UBF-binding sites in the UCE. This is thought to prevent UBF from binding to its cognate sequence and to decrease rRNA expression (Santoro and Grummt 2001). In humans, despite the fact that the CpG density in both promoter regions differ from mice (Ghoshal et al. 2004; Santoro and Grummt 2001), rRNA expression has nevertheless been shown to be epigenetically regulated (Brown and Szyf 2007). Indeed, the active portion of the rRNA promoter associated with Pol I has been shown to be completely unmethylated, while the inactive portion is almost fully methylated (Brown and Szyf 2007).

Recently, the epigenetic regulation of rRNA expression was studied in the hippocampus of suicide completers with a history of childhood abuse (McGowan et al. 2008). Analysis of the methylation pattern in the rRNA promoter (core promoter and UCE) revealed hypermethylation on 21 out of 26 CpGs, and was associated with low rRNA expression in the hippocampus of abused suicide completers compared with controls. In other words, abused subjects showed increased overall promoter methylation compared with controls, which exhibited consistently low levels of methylation. From a mechanistic point of view, it could be hypothesized that promoter hypermethylation represses the interaction of transcription factors with the DNA sequence and consequently decreases the transcriptional activity of RNA polymerase.

It should be noted that these epigenetic alterations were restricted to the hippocampus, since no group difference in rRNA methylation pattern was found in the cerebellum, a brain region that has not been primarily associated with MDD or suicide. Moreover, among the abused suicide completers, the assessment of genome-wide methylation levels did not reveal any methylation differences, further suggesting that this epigenetic alteration was specific to the hippocampus.

### *4.5.2 Brain-Derived Neurotrophic Factor*

Neurotrophic factors have consistently emerged as candidate molecules in the neurobiology of suicide. Neurotrophins are involved in neuronal survival and plasticity, and they are found throughout the brain, including the limbic system, where emotional behaviors are processed. Their alteration could underlie, at least in part, changes in plasticity observed in the brains of suicide completers as well as the

defective affect observed in depressive patients. While the major neurotrophic factors include nerve growth factor (NGF), neurotrophin 3 and 4 (NT-3/4), fibroblast growth factor (FGF), and BDNF, the latter has received most of the attention concerning the potential implication of neurotrophic factors in MDD and suicide.

In humans, low serum and brain *BDNF* mRNA levels were found in patients with major depression (Brunoni et al. 2008; Dwivedi et al. 2003; Pandey et al. 2008), and in addition, these levels were found to be reversible by antidepressant treatment (Chen et al. 2001; Sen et al. 2008; Matrisciano et al. 2009). Depressive-like behaviors have also been reported in mice following BDNF depletion (Chan et al. 2006). In rats, chronic stress and persistent pain have been shown to reduce *BDNF* expression in the hippocampus (Gronli et al. 2006; Duric and McCarson 2005), an effect that was counteracted by antidepressant treatment (Duric and McCarson 2006; Rogoz et al. 2005; Xu et al. 2006).

Recently, the epigenetic state of *Bdnf* was assessed in mice (Tsankova et al. 2006) and rats (Roth et al. 2009). In both species, the Bdnf gene has been shown to contain nine 5′ noncoding first exons with their own promoter coding for the same protein (Aid et al. 2007). The alternative splicing of these exons specifies the tissue in which *Bdnf* is expressed (Aid et al. 2007). In mice, chronic social stress was shown to decrease the expression of two specific *Bdnf* transcripts (III and IV) in the hippocampus (Tsankova et al. 2006). Similarly, lower *Bdnf* mRNA expression has been reported in the PFC of maltreated rats (Roth et al. 2009). Although similar, these transcriptional alterations were shown to be induced by different epigenetic mechanisms. In mice, low hippocampal Bdnf levels were related to higher H3K27 methylation levels in the promoter regions transcripts III and IV, while DNA hypermethylation was found in the promoter of transcripts IV and IX of maltreated rats. Interestingly, site-specific hypermethylation in maltreated rats was reported for exon IX immediately after the maltreatment regimen, while exon IV methylation gradually increased to reach significantly altered levels only at adulthood. These findings suggest that chronic stress might alter different epigenetic mechanisms than those altered by maternal maltreatment in rodents: the first leading to the compaction of chromatin in its heterochromatic state and the second blocking the binding of transcription factors to DNA both alterations decreasing transcription of BDNF.

The effect of chronic stress on *Bdnf* transcription in mice was shown to be reversed by chronic treatment with the tricyclic antidepressant imipramine, but via an indirect pathway (Tsankova et al. 2006). Indeed, chronic imipramine treatment did not reinstate H3K27 basal methylation levels, but rather decreased HDAC5 levels in the hippocampus of chronically stressed mice. This was associated with higher hippocampal levels of H3 acetylation and H3K4me in the area of *Bdnf* III and IV promoters, with both modifications related to transcriptional activation (Kouzarides 2007). Taken together, these results suggest the existence of a compensatory mechanism in the reinstatement of basal Bdnf levels by chronic imipramine treatment following chronic stress.

### 4.5.3 Tropomyosin-Related Kinase B

Tropomyosin-related kinase B (TRKB) receptor is a transmembrane receptor with high affinity for BDNF, which has been consistently linked to mood disorders and suicide (Dwivedi et al. 2003, 2009; Duman and Monteggia 2006; Kim et al. 2007). For example, microarray studies have reported lower *TRKB* expression in the PFC of depressed subjects (Aston et al. 2005; Nakatani et al. 2006), and antidepressant treatment has been shown to increase its expression in cultured astrocytes (Mercier et al. 2004).

The TRKB gene is found on chromosome 9 at locus q22.1 and has five splice variants; splice variant b T1 or TRKB-T1 is an astrocytic truncated form of TRKB lacking catalytic activity (Rose et al. 2003). Recently, a subset of suicide completers with low levels of TRKB-T1 expression in the PFC were identified (Ernst et al. 2009b). Such a pattern of expression is expected to increase predispositions to suicidal behaviors.

Analysis of the methylation pattern in the promoter of those low TRKB-T1 expressors revealed two sites where methylation levels were higher in suicide completers compared with controls. The methylation pattern at those two sites was negatively correlated with the expression of TRKB-T1 in the low expression subset of suicide completers. These results were specific to the PFC, since no significant difference was found in the cerebellum. Moreover, enrichment of H3K27 methylation associated with TRKB-T1 promoter has been reported in the same subjects (Ernst et al. 2009c). This suggests that the astrocytic variant of TRKB may be under the control of epigenetic mechanisms involving histone modifications and DNA methylation, and that these epigenetic changes are involved in suicidal behavior. These findings also support the involvement of an astrocytic component in suicide. Further studies will be necessary to identify other genes expressed in astrocytes that may be altered in suicide.

### 4.5.4 Quaking

In the brain, quaking (QKI) is expressed specifically in oligodendrocytes and is thought to be involved in cell development and myelination processes (Ebersole et al. 1996; Zhao et al. 2006). QKI expression was reported to be decreased in schizophrenia subjects, independent of neuroleptic treatment (Aberg et al. 2006; Aston et al. 2004). More recently, its expression was also shown to be reduced in cortical regions from suicide completers (Klempan et al. 2009a).

QKI gene in human is located on chromosome 6q26 and undergoes extensive splicing leading to different isoforms, each differing in the 3′ region but all exhibiting a common 5′ region (Li et al. 2002; Siomi et al. 1993). It has been recently shown that alterations in methylation were not involved in the decreased cortical expression of QKI. Indeed, methylation was very low in the promoter region of both suicide

completers and controls, which suggests that other mechanisms may be responsible for the modified expression of QKI in suicide completers (Klempan et al. 2009b). On the other hand, since the promoter region investigated was relatively small, it remains possible that other sites within the promoter control the expression of QKI. It is also possible that the altered expression of this gene is related to posttranslational mechanisms, such as microRNA.

## 4.6 Neurotransmitters and Their Receptors

### *4.6.1 GABA*

GABA is the main inhibitory neurotransmitter in the brain – it is ubiquitous and acts as a modulator of neuronal activity. It has been frequently suggested that the GABAergic system is involved in suicide (Brezo et al. 2008a; Mann 1998), for instance, the expression of numerous GABA receptor subunits has been shown to be altered in both the PFC, frontopolar cortex (FPC) and limbic system of suicide and depressed subjects (Sequeira et al. 2007; Klempan et al. 2009a; Merali et al. 2004; Poulter et al. 2008). Decreased expression of $GABA_A$ receptor subunits α1, α3, α4, and δ was reported in the FPC of suicide completers compared with controls (Merali et al. 2004). Moreover, higher density of GABA neurons was reported in several brain areas, including the hippocampus in MDD suicide subjects vs. controls (Bielau et al. 2007). In contrast, these findings have been challenged by another group reporting lower density of GABA neurons in the dorsolateral PFC of MDD suicide subjects compared with controls (Rajkowska et al. 2007).

Lower expression of $GABA_A$ receptor subunit α1 in the FPC of suicide completers has been correlated with promoter hypermethylation (Poulter et al. 2008). Indeed, hypermethylation was reported at two specific CG sites within the promoter and the transcription start site (TSS) regions in suicide completers compared with controls. Being responsible for the addition of methyl groups to DNA, it was hypothesized that aberrant activity of DNA methyltransferases (DNMT) could be responsible for the increase in methylation reported in the promoter of the $GABA_A$ receptor α1 subunit. Accordingly, a significant negative correlation between DNMT3b and methylation levels in the promoter of the α1 subunit was reported in suicide completers (Poulter et al. 2008). Given the role of DNMT3b in de novo methylation, these findings suggest that DNMT3b could be responsible for the increased methylation found in the promoter of $GABA_A$ receptor subunit α1 in suicide completers. Moreover, one of the hypermethylated sites is a putative binding site for CREB (Kang et al. 1994). All together, these findings suggest that modifications in the epigenetic machinery occurring in suicide brains could generate alterations in DNA methylation patterns. Consequently, this could block the binding of transcription factors to regulatory regions and repress the expression of genes involved in fundamental neuronal processes. This suggests that histone

modifications and DNA methylation might be altered in the brain of suicide completers with schizophrenia, however, it is also possible that such alterations might be found only in a subpopulation of schizophrenia subjects with a particular predisposition to commit suicide.

### *4.6.2 Reelin*

Reelin is a protein selectively expressed in GABAergic neurons in the cortex, hippocampus, and cerebellum (Ikeda and Terashima 1997; Pesold et al. 1998), with a major role in synaptic plasticity, learning, and memory formation (Weeber et al. 2002; Beffert et al. 2006; D'Arcangelo 2005). In the cortex and hippocampus, reelin is released from the dendritic spines of glutamatergic neurons and promotes binding and phosphorylation of NMDA receptors. This results in increased protein synthesis within dendritic spines and neuronal signaling (Dong et al. 2003).

The reelin gene maps to chromosome 7q21–22, spans through 450 kb and is composed of 65 exons (Chen et al. 2002). Its promoter region is GC – rich (75%), expanding from 1,200 bp upstream to about 200 bp downstream of the TSS (Grayson et al. 2006). A 50% reduction of reelin mRNA (Guidotti et al. 2000) and an increase in DNMT1 expression (Veldic et al. 2004) were previously reported in *postmortem* brains of schizophrenia and patients with bipolar disorder, including suicide completers. In the occipital cortex, this has been associated with site-specific hypermethylation in reelin's promoter (two sites) and in the first exon following the TSS (one site) (Grayson et al. 2005), both regions with putative CREB-binding site. Similarly, Tamura et al. (2007) found a significant negative correlation between reelin expression and methylation level at three different CG sites in reelin's promoter.

### *4.6.3 Serotonin*

The serotonergic system has been associated with MDD and suicidality (Brezo et al. 2008a; Mann 1998), with lower concentration, binding, neurotransmission, and reuptake of serotonin and its metabolites representing risk markers for suicidality and MDD (Cronholm et al. 1977; Bhagwagar and Cowen 2008). Particular attention has been given to the 5-$HT_{2A}$ receptor gene as being a major candidate in association studies of suicidal behavior (Du et al. 2001). The 5-$HT_{2A}$ receptor gene polymorphism T102C has been extensively studied and the CC genotype has been previously associated with higher scores in the Hamilton depression scale HAMD (Du et al. 2000), suicidal ideation (Du et al. 2000), and suicidal attempts (Arias et al. 2001), however, these findings failed to be replicated by other groups (Vaquero-Lorenzo et al. 2008; Li et al. 2006). The C allele does not alter the amino acid sequence,

although it may modify the secondary structure of mRNA (Arranz et al. 1995) and thus generates less transcriptionally active mRNA than the T allele (Polesskaya and Sokolov 2002). Moreover, the C allele has been associated with lower 5-$HT_{2A}$ receptor binding in postmortem suicide brains (Turecki et al. 1999).

Recently, De Luca et al. (2009) investigated C allele methylation levels in suicide and control subjects with schizophrenia, based on the hypothesis that the 102T/C polymorphism might directly influence 5-$HT_{2A}$ mRNA levels through methylation of the C allele. A trend toward lower C allele methylation levels in the PFC of suicide completers compared with controls was initially reported. On the other hand, hypermethylation was found in leukocytes from patients with a history of lifetime suicide attempts. These findings suggest that methylation levels of the C allele may be different in individuals who have committed suicide than in those who are planning or attempting suicide. The same group previously showed that the C/T allele expression ratio was significantly smaller in the PFC of suicide subjects compared with controls (De Luca et al. 2007). With their latest results, the authors suggest that the 102C allele may generate a repressor binding site, which would explain lower steady mRNA level of the C allele. Thus, methylation at this particular site could abolish the repressiveness of this site by blocking the binding of the repressor to its cognate sequence, thereby increasing 102C allele expression in the PFC of suicide subjects with schizophrenia (De Luca et al. 2009); however, functional studies are required to validate this hypothesis.

## 4.7 Concluding Remarks

Although we have increased our knowledge concerning the role of epigenetic factors in depressive disorders and suicide over the last few years, we are still far from understanding how environmental events induce specific alterations at the level of DNA, mRNA, and proteins. For now, strong direct experimental evidence in rats and indirect retrospective evidence in humans support the fact that environmental factors could alter the epigenetic mechanisms that govern expression of genes thought to be involved in the control of important neuronal processes, which, in turn, are associated with behavioral changes. The majority of studies performed to date have explored candidate genes hypothesized to be involved in the pathophysiology of major depression and suicide, but, while these studies are important, they are limited in scope. Consequently, the time has come to examine epigenetic alterations on a large scale. For instance, genome-wide high throughput comparative hybridization arrays, large scale genome-based "deep" sequencing and ChIP-based methods assessing histone modifications are now available. These large-scale studies will open the door to the systematic investigation of epigenetic effects that may be involved in suicide risk and allow the future development of novel and more targeted therapeutic strategies.

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# Chapter 5
# Epidemiology Research and Epigenetics: Translational Epidemiology of Schizophrenia

**Mary Perrin, Karine Kleinhaus, Mark Opler, Julie Messinger, and Dolores Malaspina**

**Abstract** Epigenetic processes can explain some of the epidemiological associations between environmental exposure and disease, particularly when the exposure occurs at a critical developmental stage. In this chapter, we present several epigenetic pathways associated with the risk for schizophrenia. We discuss nongenetic factors – such as paternal age, toxin exposure, and psychological stressors – which may influence human development by way of epigenetic mechanisms.

**Keywords** Environmental toxins · Imprinting · Intrauterine stress · Paternal age · X-chromosome

## 5.1 Introduction

The science of epigenetics has emerged in the last decade to study and define the molecular mechanisms that control gene expression. This field promises to illuminate some key risk pathways of complex genetic disorders, including schizophrenia. After decades of genetic studies, the risk for common human diseases remains largely unexplained. Before the Human Genome Project, scientists anticipated finding as many as 150,000 human genes. By 2001, the expected number of human genes was revised to less than 40,000, and based on the recent estimates, we now expect to count just 20,000 human genes. A related line of studies has demonstrated that humans are remarkably similar in their genetic codes, sharing 99.9% of nucleotide sequences. Human variability is therefore associated with only a 0.1% variation in nucleotide sequences. How can the great diversity among

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_5,
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individual people be explained, given so few genes and so much similarity in nucleotide sequences?

Epigenetic mechanisms provide a fresh perspective on human heredity and variation, particularly in the areas of behavior and metabolism. In classic genetic models, alterations of nucleotide sequences are predicted to influence the phenotype and determine disease risk, typically by generating greater or lesser amounts of a protein or somewhat different proteins. This is still the major focus of most genetic research. However, epigenetic mechanisms can initiate, sustain, control, and silence gene expression through DNA methylation, RNA-associated silencing, and histone modifications. Some animal studies have suggested that epigenetic information may be heritable (Roth et al. 2009a, b). Epigenetic processes are critically important for cell functioning and their marks can be altered during development. These epigenetic marks may arise during our life course or possibly through our more immediate ancestors (Mirabello et al. 2010). Most importantly, some epigenetic marks may be biologically determined in response to environmental exposure, which may then be transmitted to descendants. Epigenetic marks are metastable and may be altered through stochastic processes and gained or regained, presumably to enhance the survival of the current organism and possibly of future generations. RNA can direct epigenetic modifications to specific gene promoter regions, usually causing these regions to be silenced. Such RNA-associated silencing arises from the transcription of segments that were previously considered to be junk DNA.

This previously unknown mechanism led to a major paradigm shift, as we have learned more about this formerly obscure layer of complexity in our genome. Contrary to the "central dogma" that "DNA makes RNA and RNA makes protein," we now know that RNA can also epigenetically regulate DNA expression. RNA interference molecules are coded by the antisense strand of small interfering RNAs (siRNAs) (Anway et al. 2005; Gibney and Nolan 2010). New research in epigenetic mechanisms will reveal how and why certain genes are silenced or expressed and whether these epigenetic marks remain stable or fade over generations or disappear within a single lifetime. There are bound to be other surprises in the systems that determine human variation.

Alterations in gene expression likely occur over short periods of time in response to homeostatic and recuperative requirements and environmental stimuli, including interactions with other people (Anway et al. 2005; Morgan et al. 1999; Roth et al. 2009a, b). However, other epigenetic pathways that influence our molecular identity are more stable; there is evidence that these may be established to optimize the fit of each individual human being to his or her expected environment. The establishment of such epigenetic marks may be based on intergenerational influences, such as the exposures and challenges of our parents and grandparents and whether certain genes are maternally or paternally inherited, and on the intrauterine environment. While these events precede our birth, it appears that they may have lasting effects on our physiology by way of epigenetic mechanisms. In essence, the environment of our immediate ancestors and our own life course exposures may transform our identity at the molecular level to enhance our adaptability.

There are now scores of exciting topics in the field of the epigenetic regulation of gene expression. Epigenetic information modulates our growth and metabolism and surely influences the risk for chronic disorders such as diabetes and cardiovascular disease. As this book demonstrates, however, epigenetic processes hold particular interest with respect to the behavior and the risks for neuropsychiatric disorders. Epigenetic mechanisms powerfully explain neuroplasticity and the necessary adaptability of human behavioral repertoires.

This chapter demonstrates that data from epidemiological research can indicate possible epigenetic pathways – including intergenerational effects, prenatal programming, and later paternal age – to schizophrenia.

## 5.2 Schizophrenia

Schizophrenia is a severe neuropsychiatric syndrome with a prevalence of 0.30–0.66% and an incidence of 10.2–22.0/100,000 person years (reviewed in van Os and Kapur 2009). The symptoms typically begin in late adolescence or early adulthood, whereupon lifelong disability typically ensues. Onset is defined by the emergence of psychosis in the setting of deteriorating function and other symptoms (van Os and Kapur 2009). Before the onset of psychosis, during a prodromal period of several weeks to many years, nonspecific and variable subtle abnormalities worsen and coalesce into the classic disease features. These include alterations in the perception of reality, changes in the form and content of thoughts and speech, and social and emotional deficits including a disturbed sense of self, social dysfunction, apathy, and peculiar behavior (Perkins 2004). The symptoms of schizophrenia are often grouped into positive and negative subtypes, although there may be substantive diversity in the pathophysiology of the symptoms within these groups. Positive symptoms (so named because these phenomena occur in addition to usual experiences) include hallucinations and delusions and disorganized thinking or behavior. Negative symptoms arise from the absence of normal behaviors or experiences, including affective flattening, alogia (impoverished thinking manifested by diminished speech output or content), apathy, avolition (lack of energy and drive), and social withdrawal (van Os and Kapur 2009).

### *5.2.1 Genetic Etiology of Schizophrenia*

Early family, twin, and adoption studies showed that heritable factors were the major components of schizophrenia vulnerability. Indeed, a century of research demonstrated substantially increased morbidity risks for schizophrenia in thousands of the first degree relatives of schizophrenia probands (parents' mean, 5.6%; siblings' mean, 10.1%; and children' mean, 12.8%) compared with the general population (Gottesman and Shields 1982). These early findings remained robust against more

modern studies that directly interviewed all relatives and made operationalized diagnoses "blind" to kinship status (Goldstein et al. 1990; Pulver et al. 2004; Wolyniec et al. 1992). Since family studies cannot distinguish between genetic and environmental influences on familial aggregation, twin studies were also employed. Monozygotic (MZ) and dizygotic (DZ) twin pairs differ in their genetic endowment (sharing 100% of genetic variability and an average of 50% of genetic variability, respectively) and are typically exposed to the same familial environment. Accordingly, and in keeping with genetic hypotheses, MZ and DZ co-twins of schizophrenia probands differ in their risk for the disease. Since the initial twin studies of Luxenburger (1928), conducted over 80 years ago, the mean probandwise concordance for MZ twins are consistently threefold higher than for DZ twins (e.g., 59.2 and 15.2% in Kendler 1986). These risks are 40–60 times the risk to the general population, supporting genetic causation. Genetic studies show schizophrenia to be a highly heritable disorder with a heterogeneous clinical presentation. Although dominant, recessive, sex-linked, oligogenic, and polygenic models for schizophrenia transmission have been proposed and variably supported, research has favored the likelihood of a prominent polygenic component involving rare and common alleles, each having a very small effect on the overall population risk (Purcell et al. 2009). Recently, however, it has been suggested that highly penetrant, rare variants of recent origin, affecting neurodevelopmental pathways, may account for some of the population risk (Walsh et al. 2008).

There is an ample room for epigenetic explanations for the risk, onset, and progression of illness. For example, while the significant pairwise concordance for illness in monozygotic twins confirmed the genetic influence for the risk, it did not explain the nonconcordance in identical twins. Viewing the twin study data from the elegant perspective of epigenetics is informative. Petronis et al. (2003) made a leap forward for the field when he showed that monozygotic twins exhibit numerous epigenetic differences, which could explain their discordance. Epigenetic factors can contribute to phenotypic discordance in genetically identical organisms because these factors influence the silencing and expression of genes. They can be determined in development by individual exposures and/or subjected to addition or loss by stochastic processes – an elegant rebuttal to the warring camps of nature vs. nurture!

### *5.2.2 Nature and Nurture*

Over the history of psychiatric discourse, debates have continued as to whether mental outcomes, ranging from intelligence to temperament to schizophrenia, are better explained by "nature" or "nurture." From the new perspective of epigenetics, gene–environment interaction pathways may form or activate molecular modulators that control gene expression, for the purpose of enhancing the adaptability of the organism to an expected environment. If metastable changes in gene expression that arise from exposures (i.e., nurture) are heritable, then the boundary between

nature and nurture is obviated. In the setting where epigenetic changes can be inherited and passed on to subsequent generations, the "nurture" of one generation contributes to the "nature" of subsequent generations. Thus, nature and nurture are not distinct, and are certainly not at war.

Human disease may result from failures of epigenetic modifications, as can occur with a mutation in the DNA that precludes methylation, or from the continuance of an epigenetic modification that evolved to enhance survival in our ancestors when food was limited, but which now increases the risk for obesity and diabetes in a setting of food abundance (Gluckman et al. 2008). Likewise, epigenetic changes related to prenatal adversity may have yielded a vigilant and a hyperactive offspring, whose chances for survival were enhanced in an unstable ecosystem, perhaps with new predators. Yet in our society, the same molecular program may be associated with behavioral malfunction or mental illness.

## 5.3 Epigenetics, Fetal Environment, and Effects on the Offspring

Epidemiological studies show that several environmental factors increase the risk for schizophrenia. These exposures may act alone or in concert with variants in certain genes, or may alter epigenetic mechanisms that control gene expression. The intrauterine environment may be particularly salient for alterations in fetal gene expression. Currently, laboratory evidence is being sought to demonstrate that changes in epigenetic mechanisms are the link between the environmental exposures and the risk for schizophrenia.

### *5.3.1 Epigenetics and the Intrauterine Environment*

DNA methylation can vary due to environmental factors including nutrition, chemical exposures, or psychosocial issues (Bergman et al. 2010; Cooney et al. 2002; Pilsner et al. 2007; Roth et al. 2009a, b). Future laboratory research should continue to investigate epigenetic mechanisms that may underlie the relationship between the environment and the risk for psychosis (Rutten and Mill 2009). While epidemiologic studies do not often prove causality, translational research linking epidemiologic and basic science studies are likely to advance our understanding of both the etiology of schizophrenia and potential therapies (McGrath and Richards 2009).

There is continuing debate about the contribution of the intrauterine environment to the later risk for schizophrenia. While high rates of discordance for schizophrenia in monozygotic twins is often cited as evidence for the role of environmental factors in the etiology of schizophrenia, the discordance could potentially result from changes occurring in DNA methylation from the early

embryonal development period onwards (Schaefer et al. 2007). These epigenetic changes could plausibly be stochastic in nature and independent of the environment (Fraga et al. 2005; Petronis et al. 2003).

### 5.3.2 Epigenetic Processes, Intrauterine Exposures, and Schizophrenia

Because schizophrenia is considered to be a neurodevelopmental disease, current research is focusing on epigenetic processes in the context of the intrauterine environment. The large array of diverse fetal exposures associated with schizophrenia includes maternal infection (Brown 2006), famine (St Clair et al. 2005), preeclampsia (Cannon et al. 2002), diabetes (Cannon et al. 2002), stress (Malaspina et al. 2008), and other adversities. These disparate intrauterine events may cause schizophrenia by way of unique unrelated pathways, such as immune effects on the fetal brain, direct effects of infectious agents, compromised blood flow, decreased caloric intake, and other mechanisms (Koenig et al. 2002).

A more parsimonious expectation is that they may act through a final common pathway that is a nonspecific indicator of prenatal adversity. If so, the mechanism may involve the corticotropin-releasing hormone (CRH) and glucocorticoid hormones, which are thought to induce changes in gene expression (Cottrell and Seckl 2009; Seckl 2004; Seckl and Holmes 2007; Welberg et al. 2000). In animal studies, epigenetic changes in offspring have been linked to intrauterine exposure to endogenously or exogenously administered glucocorticoids, which are known to be associated with stress in pregnancy.

In humans, glucocorticoids from the maternal adrenal glands stimulate placental CRH production and gene expression (King et al. 2001). While CRH is well appreciated for its activity as the central regulator of the stress response in rodents and humans, it plays additional roles in human pregnancy, during which a large amount of CRH is synthesized by the placenta and secreted into the maternal and fetal circulatory systems (Mastorakos and Ilias 2003; Reis et al. 1999). CRH is a primary determinant of gestational length (Buss et al. 2009; Smith and Nicholson 2007; Wadhwa et al. 2004, 1998) and an important mediator of fetal growth and development (Ellman et al. 2008; Wadhwa 2005; Weinstock 2005). In the setting of a threatened pregnancy, either from maternal or fetal complications or from extrinsic factors, the secretion of maternal cortisol and CRH can ramp up the placental CRH production to accelerate parturition and restrict fetal growth independent of other effects of the medical complications (Hobel et al. 1999; Wadhwa et al. 2004). Low birth weight and preterm birth, both of which have been linked to schizophrenia, can result (Wadhwa et al. 2004). These actions of CRH are consistent with the hypothesis that a number of adversities influence the health and viability of the fetus, including the later risk for schizophrenia, via a final common pathway.

It is a challenge to disentangle the impact of CRH on an adverse pregnancy outcome when it arises secondarily to medical complications, which can themselves cause fetal adversity. However, if CRH mediates the risk, then severe stress and stress sensitivity alone should be associated with the risk for schizophrenia in susceptible individuals. Indeed, a number of epidemiologic studies link prenatal stress with schizophrenia risk, particularly when the stress occurs in early pregnancy (Khashan et al. 2008; Malaspina et al. 2008). Epidemiologic studies show that acute psychological trauma and nutritional deprivation in pregnant mothers from war (Malaspina et al. 2008), famine (St Clair et al. 2005), or bereavement (Khashan et al. 2008) is associated with increased risk for schizophrenia in the offspring, as well as for autism, intellectual dysfunction, and decreased language abilities (Beversdorf et al. 2005; Khashan et al. 2008; King and Laplante 2005; Kinney et al. 2008; Laplante et al. 2004, 2008; Malaspina et al. 2008; Susser and Lin 1992). The increased risk for schizophrenia depends, in part, on the timing of the insult during early pregnancy, suggesting that environmental exposures affect risk only during certain critical periods of fetal development (Khashan et al. 2008; Malaspina et al. 2008; Susser and Lin 1992).

We analyzed the effects of fetal exposure to acute maternal stress by month of gestation. The Arab/Israeli Six Day War of June 1967 was a severe psychological stressor that lasted for a month, but with no long-term nutritional deprivation or displacement for Israeli mothers. In May 1967, Egypt closed the Gulf of Aqaba to Israeli shipping, effectively beginning the war and its associated stress. The war was ended by the UN-arranged cease-fire on June 10. Our study found that the risk for schizophrenia in offspring was associated only with a maternal exposure to the stress of war in the second month of pregnancy; exposure during other months showed no effect on the risk for schizophrenia. Furthermore, the effects differed by sex, as illustrated in Fig. 5.1 (Malaspina et al. 2008), with female offspring showing a greater increase in the risk of schizophrenia in the presence of stress in pregnancy.

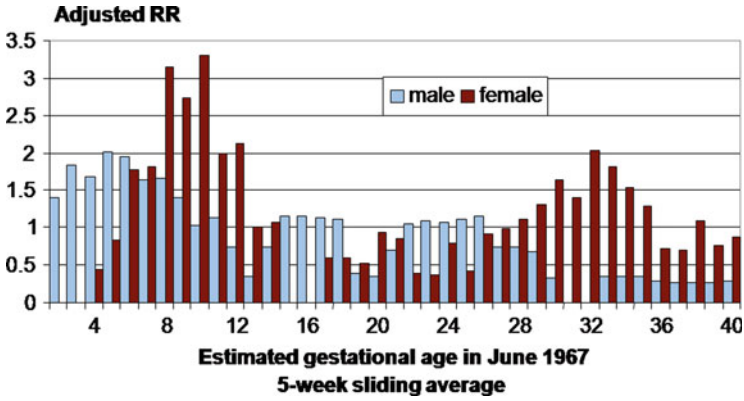


**Fig. 5.1** Schizophrenia in males and females, by estimated gestational age on June 1967. Five-week sliding average incidence, adjusted for paternal and maternal age, month of birth, low social class, and duration of marriage (Malaspina et al. 2008)

### 5.3.3 Longitudinal Epidemiological Research in Birth Cohort Studies Can Identify Possible Epigenetic Pathways

Longitudinal research in a population-based cohort is the optimal method for identifying possible epigenetic factors in epidemiological models. Disorders that are associated with environmental exposures in critical developmental periods suggest that the epigenetic mechanisms have a causal role in the pathway to disease. Our group's work on severe intrauterine stress and paternal age was conducted using data from the Jerusalem Perinatal Study. This is a population-based cohort derived from data on all births from 1964 to 1976 to residents of a defined geographic area in Jerusalem and nearby. The cohort includes core information from the birth certificate supplemented with data from multiple sources, including maternal interviews and pediatric admissions. The Ministry of Health linked the Jerusalem Perinatal Study with Israel's Psychiatric Case Registry, providing information on psychiatric morbidity. Run by the Ministry of Health since 1950 (Lichtenberg et al. 1999), the Psychiatric Case Registry contains a record of all admissions to psychiatric hospitals or psychiatric wards within general hospitals, and admissions to day facilities for psychiatric treatment. It includes dates of admission and discharge and a single discharge diagnosis for each episode, assigned by a board-certified psychiatrist. These diagnoses are coded with the International Classification of Diseases (ICD); codes from earlier years have been updated to the tenth revision and those for psychotic disorders have been validated (Weiser et al. 2005).

In our research, we defined schizophrenia broadly so as to include discharge diagnoses of schizophrenia, schizotypal disorder, delusional disorders, nonaffective psychoses, and schizoaffective disorders, hereafter called "schizophrenia-related disorders" (ICD-10; codes F20-29).

Of the original 92,408 births in the cohort, the identities of 90,079 (97.5%) were verified through Israel's population registry and their vital status ascertained. Approximately, 0.7% were lost to follow-up due to changes in identity number (e.g., adopted, formally emigrated, or in witness protection programs). The remaining 1.8% untraced included 37% who had been born to unmarried mothers (likely to have been adopted) and 12% whose mother had no ID number (likely to have been diplomats or foreign exchange students). There were 861 cases of schizophrenia-related diagnoses in the traced cohort, and 761 individuals with "other" causes of psychiatric hospital admission. The median age at the first hospital admission was 22.8 (range 5–39) for the schizophrenia-related diagnoses and 21.4 (range 5–40) for the other diagnoses. The annual incidence of first hospital admissions in individuals with schizophrenia-related diagnoses was 0.02/1,000 by ages 8–9, increasing to 0.9/1,000 by ages 19–20, 0.5/1,000 by ages 29–30, and during the next decade 0.01/1,000. The life-table estimate of cumulative incidence of schizophrenia was 1.2% by ages 39–40.

This cohort was established by farseeing investigators. It now provides 28–40 years of follow-up from pregnancy and birth through the age of risk for adult

diseases including schizophrenia. This rich resource of prospectively collected exposure data permits powerful and precise studies of environmental influences in critical developmental periods. Since the Jerusalem Perinatal Study contains information on the both maternal and paternal grandfathers, parents, and offspring, it allows for an intergenerational view along with intrauterine environment and life course perspectives.

### *5.3.4 Confirming Stress-in-Pregnancy Effects in Translational Studies*

Animal studies have corroborated findings of epidemiologic studies that the intrauterine environment is crucial to adult health, and have also demonstrated that prenatal nutrition (e.g., folate and neural tube defects) (Fleming and Copp 1998) and other environmental factors (Zhong et al. 2010) can affect neurodevelopment. For instance, mice whose mothers received a choline supplemented diet during pregnancy showed significantly improved sensory inhibition (a biomarker for schizophrenia) compared to control mice; they also showed an increase in α-7 receptor numbers in both the CA1 and the dentate gyrus regions of the hippocampus (Stevens et al. 2008). Nutritional supplementation during gestation resulted in permanent improvement in a physiological task that is deficient in animal models of schizophrenia, suggesting that the intrauterine environment might also ameliorate the risk for schizophrenia.

Obstetric complications may also influence the risk for schizophrenia. A study found that certain single nucleotide polymorphisms (SNPs) in four genes regulated by hypoxia are involved in neural vascular functioning interact with serious obstetric complications to influence the risk of schizophrenia. Offspring with "risk" alleles at these SNPs and the history of obstetric complications had a higher risk of schizophrenia than those without the polymorphisms (Nicodemus et al. 2008). These findings correlate well with epidemiologic findings that obstetric complications, including preeclampsia, hemorrhage, maternal sepsis at childbirth, and manual extraction of the baby (Byrne et al. 2007) are associated with increased risk of schizophrenia.

Building on epidemiologic studies that associate stress early in pregnancy with schizophrenia (Brown 2006; Malaspina et al. 2008; St Clair et al. 2005), researchers analyzed the effects of intrauterine exposure to glucocorticoids during early gestation. Pregnant rats that had been injected with synthetic glucocorticoid during early pregnancy delivered offspring that showed decreased juvenile social play, a blunted acoustic startle reflex, increased prepulse inhibition of startle, and reduced amphetamine-induced motor activity, which are animal behaviors considered relevant to schizophrenia. In addition, dams, which were treated, exhibited increased milk ejection bouts during nursing. The frequency of milk ejections significantly interacted with the effects of dexamethasone on play behavior and acoustic startle reflex.

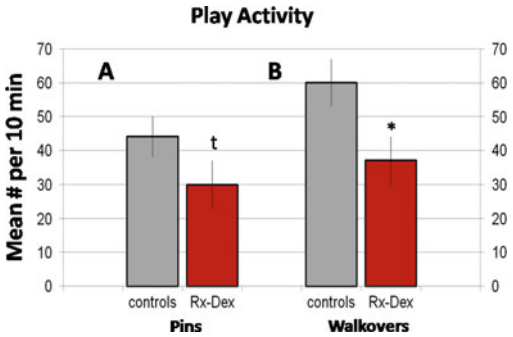


**Fig. 5.2** Social play behaviors in juvenile offspring of dams treated with saline (CON; $n = 6$; *gray bars*) or DEX ($n = 5$; *red bars*). *Bars* show litter means (±SEM) for the number of pins (A) and walkovers (B) per a 10-min period of interaction between two same-sexed littermates. DEX offspring showed decreases in play behavior. $^{*}p < 0.05$, $^{t}p = 0.16$ (Kleinhaus et al. 2010)

Both intrauterine conditions and postnatal maternal behavior likely contributed to the effects of early environment on an increase in schizophrenia-related behaviors as demonstrated in Fig. 5.2 (Kleinhaus et al. 2010).

Prenatal stress has been shown to influence hippocampal development in a dose-dependent manner. Mild stress of short duration enhances its development, whereas long-lasting and severe stress disturbs its development (Fujioka et al. 2006). Although prenatal stress reduces hippocampal cell proliferation throughout life, as well as cell survival and differentiation (Lemaire et al. 2006), these effects can be completely counteracted by increased infantile stimulation consisting of postnatal handling (Lemaire et al. 2006). Rats that were prenatally stressed also have decreased levels of 5-HT1A immunobinding in the ventral hippocampus as compared with controls (Van den Hove et al. 2006). Rat pups whose dams were stressed during pregnancy have more anxiogenic behavior and impaired spatial learning. Adrenalectomy of the pregnant dams precluded these effects on the pups, suggesting that the high levels of corticosterone secreted by pregnant rats in response to stress mediates the intrauterine stress effects on the developing fetal brain (Zagron and Weinstock 2006).

Epigenetic mechanisms may certainly underlie these environmental effects on the risk for aberrant behavior. A rat model of maltreatment in the first week of life showed increased DNA methylation of the *BDNF* gene in the adult prefrontal cortex. Even the offspring of the females that had experienced the maltreatment regimen showed increased *BDNF* methylation, which suggests that early environment can trigger a heritable epigenetic change (Roth et al. 2009a). The molecular mechanism potentially perpetuates changes in gene expression and behavior throughout the lifespan of the stressed female and into the next generation (Roth et al. 2009a, b). *BDNF* has also been linked to schizophrenia (Weickert et al. 2003). Despite these changes in *BDNF* methylation, it is not yet established whether epigenetic regulation of gene expression is a common underlying mechanism for the link between environmental factors and schizophrenia.

## 5.4 Genomic Imprinting

The phenomenon of genomic imprinting is ancient, having arisen in a common ancestor to marsupials and eutherian mammals over 150 million years ago (Killian et al. 2000). The evolutionary implications of this mechanism are intriguing. Imprinted genes date back to the branching of the evolutionary tree about 180 million years ago, when live births began for placental mammals. As it is true for most sex differences in mammals, this mechanism may have arisen to account for the differential contribution of resources by the mother and father to the offspring of the next generation. Sex differences are commonly related to sexual reproduction, and the reciprocal silencing and expression of genes from one parent or the other is no exception. The roles of male and female differ with respect to the parturition and nurture of offspring. Likewise, the epigenetic complement of sperm and egg inherited by the young serves the paternal and maternal investments by optimizing the survival and reproduction of the young.

Genomic imprinting of genes runs counter to the usual expectation of biallelic inheritance and expression. Imprinted genes are haploid, as only a single allele is expressed. Such genes are susceptible to environmental mutagens or random mutations, as there is no alternative allele. We have proposed that these mechanisms may be particularly sensitive to later paternal age (Malaspina et al. 2001). Errors in erasure or reestablishment of these imprinting patterns may lead to defective gene expression profiles in the offspring. Human imprinted genes have a critical role in the growth of the placenta, fetus, and central nervous system; in behavioral development; and in adult body size. Imprinted genes are essential for intrauterine development through parturition.

### *5.4.1 An Imprinting Mechanism*

Imprinted genes generally, but not always, reside in clusters, which include both paternally and maternally imprinted genes. The clusters are about 1 Mb and contain a noncoding RNA gene whose product helps to regulate the imprinting of nearby genes. Imprinting control regions, called differentially methylated regions (DMR), coordinate the imprinting of genes in these clusters. During gametogenesis, the methylation marks of the previous generation (the grandparents of the offspring) are erased and subsequently re-established. Though this process is not well understood, there are several proposed mechanisms to explain it [see review in Weaver et al. (2009) for more detail].

In peripheral blood cells, the insulin-like growth factor 2 (*IGF2*) gene is silenced on the maternal allele and expressed from the paternal allele (Feinberg and Tycko 2004). The mechanism involved in this silencing or inactivation is complex, and involves the pattern of methylation at a nearby locus. *IGF2* is found in an imprinted region on chromosome 11 that also includes *H19,* a putative tumor suppressor gene,

and several differentially methylated regions (DMR). A DMR 2–4 kb upstream of *H19* contains a *CCCTC*-motif binding site for the CTCF protein (Lewis and Murrell 2004). The maternal DMR is unmethylated and accessible to CTCF, which acts as an insulator, preventing the promoters of *IGF2* from accessing the endodermal enhancers, thus silencing the maternal copy of *IGF2*. Methylation of the DMR on the paternal allele prevents the binding of CTCF, leading to its expression (Murrell et al. 2004).

Imprinting is tissue-specific, maybe even cell-specific, and may differ by the stage of development (Davies et al. 2005). For example, *IGF2* is monoallelically expressed in all tissues except the brain and the liver, where it is biallelically expressed.

Errors in imprinting may occur at any point during erasure, establishment, or maintenance of an imprint. Loss of imprinting occurs when a previously silenced maternal or paternal allele is partially or completely expressed. Loss of imprinting of *IGF2* has been consistently recognized as a risk factor for colon cancer; it is detected in blood and tumor tissue (Cruz-Correa et al. 2004; Cui et al. 2003; Cui et al. 2002). In a percentage of individuals with Angelman and Praeder-Willi (PWS) syndromes, there is a loss of gene expression due to the failure to erase in the parental germline and the imprint established in the grandparental germline (Horsthemke and Buiting 2006). Though both these syndromes are associated with behavioral, cognitive, and neurologic impairment, in the case of PWS, 5–10% of cases experience schizophrenia-like psychotic symptoms (Davies et al. 2001). Epigenetic dysregulation in the parental gametes or in the fetus could therefore influence the gene expression and function of the placenta, which would likely have a substantial impact on fetal development.

## 5.5 Paternal Age-Related Schizophrenia

While siblings receive similar genetic contributions from their parents, their epigenetic profiles may vary substantially. Changes may occur in sperm and egg cells due to aging or environmental exposures of the parents and differing intrauterine and postnatal environments. It is possible that these differences can alter behavior. We showed that the risk of schizophrenia continues to linearly increase with advancing paternal age, which helps to explain the effect of birth order on schizophrenia within families (Malaspina 2001).

### *5.5.1 De Novo Mutations and Later Paternal Age*

We have speculated that the maintenance of schizophrenia in the population, despite the reduced fecundity of affected individuals, might be explained by the

replenishment of disease susceptibility genes through new mutations. If so, then the risk for schizophrenia is expected to be related to paternal age, as this is the major source of de novo mutations in humans and other mammals. This fact is explained by the constant cell replication cycles that are ongoing in spermatogenesis. Following puberty, spermatogonia undergo some 23 divisions per year. At ages 20 and 40, a man's germ cell precursors will have undergone about 200 and 660 such divisions, respectively. By contrast, oogonia undergo only a dozen or so cell divisions, predominantly during the mother's fetal life. During a man's lifetime, the constantly dividing spermatogonia are vulnerable to random errors, DNA damage from toxins, and other mutations. These lead to errors in spermatogonia that accumulate in expanding clones as men age (Crow 1999).

### *5.5.2 Imprinting as a Mechanism Linking Paternal Age to the Risk for Schizophrenia*

Advancing paternal age could also plausibly involve epigenetic mechanisms (Brown et al. 2002; Byrne et al. 2003; Dalman and Allebeck 2002; El-Saadi et al. 2004; Malaspina et al. 2001; Sipos et al. 2004; Tsuchiya et al. 2005; Zammit et al. 2003). Paternal age has been shown in repeated studies to be a strong risk factor for schizophrenia, second only to family history of the disorder.

There are several characteristics of imprinted genes that make them reasonable candidates for schizophrenia vulnerability. Paternally and maternally expressed genes are both necessary for embryogenesis (Surani et al. 1990), playing greater roles in placental and embryo development, respectively (Kato et al. 1999). The influence of paternal genes in the placenta may represent a mechanism for the father to ensure that his offspring derives adequate resources from the maternal in utero environment, even if it may be in the best interest of the mother to limit these resources (Iwasa 1998).

Imprinted genes play a key role in brain development, leading to lasting changes in cognition and behavior (Isles and Wilkinson 2000; Keverne et al. 1996). In fact, it has been reported that among males but not females, average DNA methylation of the *IGF2*, a paternally expressed gene is significantly correlated with brain weight (Pidsley et al. 2010). There appears to be a neuroanatomic localization pattern for the expression of certain imprinted genes in mice: the paternal or maternal allele expression patterns correspond to the limbic and neocortical regions, respectively (Allen et al. 1995; Keverne et al. 1996). The conceptualization of schizophrenia symptoms as deriving from an imbalance or modulatory disturbance between these regions (Weinberger et al. 1992) might be pertinent to these expression differences. In addition, genes for several neurotransmitters implicated in schizophrenia may be imprinted, including those for the serotonin 2A receptor, the dopamine 3 receptor, and several GABA A receptors (see Meguro et al. 1997; Petronis et al. 2000).

## 5.6 Imprinting and the X-Chromosome

There is strong circumstantial evidence that there are imprinted genes on the X-chromosome. This evidence includes studies in women with Turner's syndrome. Additional support comes by way of epidemiologic studies, which are also discussed below. Further, we will look at the paternal X-chromosome as it may play a role in schizophrenia. We will also discuss skewed X-chromosome inactivation and schizophrenia.

### *5.6.1 Evidence Supporting the Role of Imprinted Genes on the X-Chromosome*

Although several imprinted genes on the X-chromosome have been detected in mice (Kobayashi et al. 2010), as of yet, none have been found on the human X-chromosome. A study by Susan Harlap investigated whether imprinted genes may play a role in schizophrenia (Harlap et al. 2009). In this study, the authors examined the association between schizophrenia in the offspring and birthplace of the maternal and paternal grandfather. The study reported that a *paternal* grandfather from Romania or Hungary increased the risk of schizophrenia in the offspring [RR 1.9; 95% confidence interval (CI) 1.3–2.8 and RR 1.6; 95% CI 1.0–2.6, respectively] whereas a *maternal* grandfather from Romania or Hungary reduced the risk of schizophrenia in the offspring (RR 0.5; 95% CI 0.3–0.8 and RR 0.4; 95% CI 0.2–0.8, respectively). The increased risk associated with having a paternal grandfather from Romania or Hungary was more apparent in female offspring than in male offspring, whereas the decreased risk associated with a maternal grandfather from these countries was similar in males and females. The authors posited that the sex differences reported in this study could be explained by an imprinted locus on the X-chromosome.

### *5.6.2 Other Research Suggesting the Presence of Imprinted Genes on the X-Chromosome*

On the basis of studies in women with Turner's syndrome (45, X), many genes related to neurocognition and social function are thought to lie on the X-chromosome. Women with Turner's syndrome have a constellation of symptoms such as short stature, neurocognitive and social function decrements, and failure to undergo puberty due to ovarian failure. Several studies have suggested that the constellation of symptoms in women with Turner's syndrome may differ between women with a maternal X (Xm) and a paternal X (Xp) chromosome. Some studies have noted that women with an Xp have better social and executive function than Xm women

(Skuse et al. 1997). Others have noted that there is strong correlation with cardiovascular disease in Xm women but not in Xp women (Chu et al. 1994). Another more recent study reported morphological differences in superior temporal gyrus gray matter between Xm and Xp women (Kesler et al. 2003). The results of these studies strongly suggest the presence of imprinted genes on the X-chromosome.

### *5.6.3 Skewed X-Chromosome Inactivation and Schizophrenia*

Since women receive an X-chromosome from each parent, they have both a maternal (Xm) and a paternal (Xp) X-chromosome. One X-chromosome is silenced in early embryogenesis in each cell line to maintain dosage compensation with males who have only one X-chromosome (46, Xm Y). In women, silencing of one X-chromosome is usually a random process resulting in 50% of cells with an Xm chromosome and 50% of cells with an Xp chromosome actively transcribed. Deviations from the expected 50:50 ratio of paternal to maternal X-chromosome silencing appear to be relatively common in females. More than 30% of women have ratios of 75:25 or more extreme distributions (Kim et al. 2004; Kristiansen et al. 2003; Lanasa et al. 1999; Struewing et al. 2006), though only 1–10% has ratios of 90:10 or more extreme distributions (Kim et al. 2004; Lanasa et al. 1999). Random inactivation is considered protective if one allele has a deleterious mutation. However, skewed inactivation can lead to a functional loss of heterozygosity (Buller et al. 1999) resulting in predominant expression of a deleterious mutation or other genetic or epigenetic error (Hedera and Gorski 2003; Kinoshita et al. 2004; Parolini et al. 1998; Pegoraro et al. 1997; Valleix et al. 2002).

Through the study of X-linked retardation syndromes and Turner's syndrome, it is thought that many loci on the X-chromosome are related to cognition and social functioning. A study by Rosa et al. (2008) reported that differential methylation of the X-chromosome in female monozygotic twin pairs was greater in discordant twin pairs than in concordant twin pairs for bipolar disorder. The results were not significant for schizophrenia. A study is currently being conducted in which it is hypothesized that extreme skewing of 90:10 will be more common in affected sisters than in their nonaffected female siblings. Among those with skewed X-inactivation, it will be determined whether Xp or Xm is preferentially actively transcribed in both affected and nonaffected sisters.

### *5.6.4 The Paternal X-Chromosome*

Sex differences in the age of onset, symptoms, and prognosis are consistently recognized in large groups of patients with schizophrenia. One of the obvious differences between males and females is that only females receive a paternal X-chromosome. It is now becoming well established that important epigenetic

changes occur in the paternal X-chromosome through gametogenesis, so later paternal age could conceivably influence the efficiency of the epigenetic processing of the paternal X-chromosome (reviewed in Zamudio et al. 2008). If so, then a greater risk for paternal age-related schizophrenia (PARS) might be observed for females.

We conducted research to determine the influence of advanced paternal age on the risk of schizophrenia in offspring based on the sex of the offspring and their relation to any other first-degree family members who were hospitalized with schizophrenia (Perrin et al. 2010). Among male and female offspring of fathers greater than 35 years of age, mothers diagnosed with schizophrenia conferred the highest overall risk of schizophrenia to their offspring, as has been reported. However, there were marked differences in the risk for schizophrenia in the female and male offspring of older fathers. Sisters of affected females born to older fathers had an almost ninefold increase in their risk of schizophrenia (95% CI 3.9–19.8) compared to the population. By contrast, for the brothers of affected males born to older fathers, the risk of schizophrenia was similar to that of male siblings born to younger fathers.

All female siblings inherit the same paternal X-chromosome. Our results suggest that aberrant epigenetic processes in the paternal X-chromosome and accumulated genetic mutations in the constantly replicating male germline as paternal age advances may increase the risk of schizophrenia in female offspring.

## 5.7 Environmental Toxins and the Paternal Germline: Epigenetic Effects

Epigenetic changes in sperm from toxic exposures are likely to be of huge public health significance. These effects may be magnified in men of advancing age. Epigenetic changes over the life course in animal studies are consistent with changes in DNA methylation with a trend toward global hypomethylation of repetitive sequences and proto-oncogenes, along with gene-specific hypermethylation increases with age (Ahuja et al. 1998; Wilson and Jones 1983; Wilson et al. 1987). Nonetheless, methylation status fluctuates over the course of development according to cell and tissue type, developmental stage, and experimental conditions. Age-related changes in sperm DNA methylation have not been as carefully studied, although hypermethylation of sequences in sperm ribosomal RNA loci in older animals was reported by Oakes et al. (2003). Both endogenous and exogenous mechanisms have been suggested for age-related changes in methylation.

Exposure to chemicals that inhibit methylation enzymes (e.g., heavy metal exposure such as nickel; Chen et al. 2006) may have long-term consequences, since DNA remethylation processes may be incomplete or prone to error. One study of monozygotic twins reports that epigenetic differences increase over time, providing further

support for the theory that the environmental exposures over time alter methylation patterns (Fraga et al. 2005).

While both human and animal studies demonstrate changes in sperm chromatin structure and stability with age, there is a lack of consensus on the nature of the changes and the degree to which these are related to aging. In a rat model, Zubkova et al. (2005) found that sperm chromatin stability was nearly equivalent in young and older rats under normal conditions. However, in the older rats, sperm chromatin was much more susceptible to oxidative stress, causing decreased stability and increases in single- and double-strand breaks. Clinical studies also suggest that stability is decreased in aging men. Using the comet assay to detect single- and double-strand breaks in DNA, Singh et al. (2003) reported a positive correlation between age and DNA fragmentation levels. Measures of reproductive health in male rodents consider pathology and outcome of proximal (pathology of testicular tissue and direct microscopic examination of spermatogenic cells), intermediate (sperm concentration, motility, and morphology), and distal processes (mating behavior and success, litter number, weight, and viability). In studies of lead-induced reproductive toxicity, there are inconsistencies in the extent to which distal vs. proximal and direct vs. indirect effects are reported (Apostoli et al. 1998; Mangelsdorf et al. 2003). Some of these differences may be related to interspecies and interstrain differences in toxicokinetics due to genetic differences.

Despite ongoing controversy regarding the precise level of exposure at which effects may be apparent in a given species or strain, there is consensus that high-level lead exposure negatively affects male reproductive health and may be responsible for reduced male fertility. Stowe and Goyer (1971) were among the first to report that mating behavior may be altered in lead exposed rats, as well as the likelihood of fertilization. Silbergeld et al (2003) found that male rats exposed to lead through their drinking water (average BPb levels of 60 μg/dL) were less likely to successfully fertilize unexposed females. In contrast, Nelson et al. (1997; see later) report no effects on fertility in rabbits (at levels of 80 μg/dL), although the viability of offspring at very high levels (110 μg/dL) was reduced.

The importance of fertility in the context of neurodevelopmental disorders has been demonstrated in work by our own group on time-to-pregnancy and risk of schizophrenia in offspring (Opler et al. 2010).

Using data from the Jerusalem Perinatal Study, postpartum interview data on the number of months required for a couple to conceive were analyzed. Compared with offspring conceived in less than 3 months, the unadjusted relative risks (RR) of schizophrenia associated with conception times of 3–5, 6–11, and 12+ months were 1.10 (95% CI 0.62–1.94), 1.41 (95% CI 0.79–2.52), and 1.88 (95% CI 1.05–3.37) with $p$ for trend = 0.035. It should be noted that in such studies, it is difficult to attribute time-to-pregnancy changes to either male- or female-related factors. However, these findings suggest that factors associated with fecundability, either male or female, may contribute to the risk of schizophrenia.

In vitro studies have shown that lead and other heavy metals may interfere with DNA binding to protamines, small proteins critical both to sperm chromatin stability, and for condensation/decondensation events during fertilization. Foster et al. (1996)

studied the effect of lead on sperm chromatin in cynomolgus monkeys and found changes in chromatin structure at exposure levels below the limit generally thought to have fertility effects in humans. Results from both in vitro and in vivo studies (Hernandez-Ochoa et al. 2006) have suggested that lead has differential effects on chromatin condensation depending on timing and dose, i.e., chromatin of immature spermatocytes that have not completed postmeiotic processing appears to be *less* condensed in lead-exposed animals. By contrast, chromatin from more mature spermatocytes that have undergone late-stage processing including incorporation of protamines appears to be *more* condensed in lead-exposed animals. The authors postulate that the negative effects on fertility are primarily due to "overstabilization" of disulfide bonds in protamines.

### *5.7.1 Heavy Metals, Solvents, Epigenetics, and Outcomes in Offspring*

Many DNA methyltransferases are zinc-dependent, making them potential targets for toxicity following exposure to lead and other heavy metals. Shiao et al. (2005) demonstrated that the specific gene coding for the promoter for 45s ribosomal RNA in mice is altered in the sperm of mice that were exposed to Chromium(III) [Cr(III)], which is known to be a transgenerational carcinogen. Key events in DNA methylation, including methioninesynthase activity have been shown to be affected by chemical exposures such as lead, ethanol, mercury, and aluminum (Waly et al. 2004).

There is some evidence to suggest that epigenetic mechanisms might mediate behavioral and other outcomes in offspring from heavy metal-exposed males. Nelson et al. (1997) demonstrated abnormalities in exploratory behavior, novelty seeking, and the rates of physical activity in the offspring of male rabbits exposed to high lead levels of 40 μg/dL or more. Statistically significant reductions in activity were seen at postnatal day 25 in offspring with paternal exposures of lead at 40 and 80 μg/dL. Both maternal and paternal lead exposure may influence offspring behavior (Brady et al. 1975), including spatial learning measured by increased swimming times in water maze tests. Offspring groups that had both parents exposed to lead demonstrated the highest level of impairment, with significantly longer swim times than either maternal or paternal exposure separately.

Researchers have also examined the growth and development of hippocampal neurons in the brains of animals paternally exposed to lead (Silbergeld et al. 2003). Hippocampal cells were removed from neonatal brains of the offspring of lead-exposed sires (postnatal day 1), and cultured; after 7 days, surviving cells (largely pyramidal) were subjected to morphological analysis. Cultures from paternally exposed animals showed two principal differences from controls: larger cell bodies and increases in the total number of cells with "higher order" branching (i.e., increased numbers of dendrites per axon). The authors suggest changes in timing and regulation of pruning may be responsible for observed behavioral findings.

From a public health perspective, exposure to epigenetically active compounds should be of concern in men of reproductive age (Sharpe 2010). Studies by Opler et al. (2004, 2008) have suggested a link between second and third trimester lead exposure and risk of schizophrenia in adulthood. While certain airborne pollutant levels, for example, lead levels, have fallen in the US since the 1970s, occupational settings still offer the opportunity for exposure. Although no longer sold commercially to the public, tetraethyl lead is used as a gasoline additive in military engines (particularly aircraft) and has only been recently removed from gasoline at NASCAR sporting events. Concern regarding exposure levels for individuals working in these settings is justifiable, particularly since there is an overrepresentation of men entering their peak reproductive years. A study of blood lead levels among a sample of NASCAR employees shows that up to 40% may have elevated blood lead levels (O'Neil et al. 2006).

Our group previously reported that paternal occupation as dry cleaner was associated with a threefold (95% CI 1.0–9.3) increased risk of schizophrenia in the offspring (Perrin et al. 2007). Tetrachloroethylene, a volatile aromatic halogenated hydrocarbon, has been used as a dry cleaning solvent for several decades. It is absorbed readily in adipose tissue and the brain is a target organ. It is primarily excreted through exhalation exposing others to contamination and it is also excreted in breast milk. In animal studies, intrauterine exposure to tetrachloroethylene has been reported to reduce postnatal levels of dopamine and acetylcholine (Nelson et al. 1979), whereas postnatal exposure caused disruption of habituation behaviors at 60 days (Fredrickson and Richelson 1979). Exposure to tetrachloroethylene in humans was found to disturb neuronal processing and alter perception of contrast (Altmann et al. 1990). Reproductive effects of tetrachloroethylene exposure have been reported to include spontaneous abortions in female workers (Ahlborg 1990; Doyle et al. 1997; Kyyronen et al. 1989; Windham et al. 1991), reduced sperm quality in male workers (Eskenazi et al. 1991b), and infertility or prolonged conception time in their wives (Eskenazi et al. 1991a). As men and women in occupational settings are likely to be of reproductive age, these types of exposures become increasingly relevant to the health of their future offspring.

## 5.8 Conclusion

Breakthrough research in epigenetics has offered new insight into the ways in which disposition to mental illness is transmitted, the biological nature of the inherited factors, and the mechanisms by which these genetic factors interact with environmental determinants. Recent advances in epidemiology have created a new and more promising context for epigenetic discovery. Prenatal and life course environmental exposures associated with schizophrenia risk, including later paternal age and prenatal adversity, could act by altering epigenetic information. Epigenetic influences on behavior may extend from the exposures of earlier generations, to those in the womb and perhaps throughout the life course.

**Acknowledgments** We would like to thank Dr. Susan Harlap and Benjamin Barasch for reviewing this manuscript and providing many insightful comments.

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# Chapter 6
# Environmental Studies as a Tool for Detecting Epigenetic Mechanisms in Schizophrenia

**Wim Veling, L.H. Lumey, Bas Heijmans, and Ezra Susser**

**Abstract** Epigenetic mechanisms may play an important role in the etiological pathways of schizophrenia. Since the epigenetic status of the genome partly depends on environmental factors in pre- and postnatal environments, exposure to such factors should be taken into account in epigenetic studies of schizophrenia. Prenatal famine and childhood ethnic minority status have been identified as environmental risk factors for schizophrenia. These exposures can be used to investigate epigenetic effects on schizophrenia, since environmental exposure can be measured with sufficient precision, homogeneously exposed populations are available for study, and plausible biological pathways have been suggested (albeit less specific for migration). This chapter shows that epidemiological studies of famine and migration can help to detect epigenetic mechanisms in schizophrenia, by comparing the epigenome of exposed and unexposed schizophrenia cases and controls. The results of the comparisons will be different depending on the mechanism involved in the interplay between environment and epigenome. If these epidemiological designs are not applied, the overall result of epigenetic schizophrenia studies may well continue to be inconclusive.

**Keywords** Environmental risk factors · Epidemiology · Epigenetics · Famine · Migration · Schizophrenia

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_6,
© Springer-Verlag Berlin Heidelberg 2011

## 6.1 Introduction

There is no doubt that schizophrenia has a strong genetic basis (Sullivan et al. 2003). In the 1990s, hopes that traditional linkage studies could identify major genes for schizophrenia were ignited by some exceptional findings for other complex disorders. Perhaps, most notable was the strong association between APOE genetic alleles and Alzheimer's disease, which was originally identified via traditional linkage studies (Kehoe et al. 1999). In the last decade, we have witnessed enormous progress in the creation of novel methods for associating variation in genes to risk for complex disorders. The advent of genome-wide association studies (GWAS), in particular, was a significant development that fueled new expectations. However, the results obtained from GWAS of schizophrenia have been quite disappointing, as they have been inconsistent and difficult to interpret (Manolio et al. 2009). A continuously updated online database of genetic association studies currently lists over 900 different genes and more than 8,000 different polymorphisms that have all been studied in relation to schizophrenia (Allen et al. 2008). Associations with common alleles generally have small effect sizes, are difficult to replicate and, thus far, rare genetic variants with larger effects only explain a small proportion of the heritability of schizophrenia (Manolio et al. 2009). Other research strategies, extending beyond the current GWAS approach, may therefore be needed. Next generation sequencing-based studies, which include whole genome sequencing among other approaches, may provide the field of schizophrenia research with a much-needed advance. Recent reviews have raised hopes that the "missing heritability" will be revealed by these sophisticated methods (Manolio et al. 2009) – this may be true, to some extent, given the enormous power they will confer in gene discovery, although the vast number of variants will make it immensely difficult to identify those which are causal. Another strategy could involve a reappraisal of (pedigree-based) linkage studies that have sufficient power to detect the effects of rare variants, that is, the situation of genetic heterogeneity where different mutations in different individuals at the same locus (gene) contribute to disease. Similar methods have failed in the past, not due to inherently flawed design, but because the studies were too small and underpowered.

Another development that may advance our understanding of the genetic architecture of schizophrenia has been in the field of epigenetics, which refers to mitotically heritable modifications of DNA that do not involve a change in DNA sequence. One advantage of epigenetics is its potential to integrate studies of the genetic and environmental causes of schizophrenia, thereby achieving more powerful designs. The substantially lower discordance rate of schizophrenia in monozygotic (MZ) twins compared with dizygotic ones argues for a strong genetic component, and also allows for a significant role of environmental factors in the development of this complex disease (Oh and Petronis 2008). In this regard, several risk factors that implicate preconceptional, prenatal, or early childhood exposures have been consistently related to schizophrenia, including paternal age at conception, early prenatal famine, urban birth, and migration (especially in early childhood, see later) (Van Os

and Kapur 2009). While some of these associations are likely to be causal, the mechanisms by which they are linked to schizophrenia are still largely unknown. Epigenetic mechanisms may mediate the effects of such risk factors, as the epigenetic status of the genome can be modified in response to the environment during embryonic growth, and probably also in the early years of life (Heijmans et al. 2009). Preliminary evidence suggests that epigenetic differences may be related to schizophrenia (Abdolmaleky et al. 2006; Mill et al. 2008), but these epigenetic studies have not yet included environmental exposures.

To determine the contribution of epigenetic components in the interplay between genes and environment, we must study populations that have been homogeneously exposed to well-defined and meticulously measured environmental exposures (Manolio et al. 2009). In this chapter, we discuss two environmental risk factors for schizophrenia in the light of epigenetics. We present epidemiological evidence of a relationship between schizophrenia and prenatal famine and migration. We then argue that the epidemiological findings are consistent with epigenetic mechanisms, and explore evidence of pathways linking these environmental exposures to schizophrenia via epigenetic effects. Finally, we propose ways to further test these hypotheses and argue that this may advance knowledge of both genetic and environmental causes of schizophrenia.

## 6.2 Prenatal Famine and Schizophrenia

### *6.2.1 Epidemiological Findings*

Tragic historical events in the Netherlands and China set the stage for investigation of the effects of famine on schizophrenia and other health outcomes. Three studies have reported a link between periconceptional or early gestational exposure to starvation and a risk of schizophrenia in offspring (St Clair et al. 2005; Susser et al. 1996; Xu et al. 2009). The first of these studies was based on the Dutch Hunger Winter during World War II. In October 1944, the Nazis blocked food supplies to the Western part of the country in response to the activities of Dutch resistance groups. The famine ended abruptly with the liberation of the Netherlands in early May 1945. Early studies found an increased risk of neural tube defects among individuals who had been conceived at the height of the famine (Brown and Susser 2008), suggesting that the early gestational period is an important risk window for neurodevelopmental insults. This finding supported the plausibility of prenatal famine as a cause of schizophrenia, since a disturbance in early neurodevelopment has also been implicated in the etiology of this disorder. When the risks for schizophrenia were compared between the birth cohort that had been exposed to prenatal famine and unexposed cohorts born in surrounding years, it was found that periconceptional or early gestational, but not later exposure to famine was associated with a relative risk for schizophrenia of 2.0 (95% confidence interval [CI] 1.2–3.4) (Susser et al. 1996).

Recently, these findings have been replicated in two large studies in China. After the initiation of the Great Leap Forward in the late 1950s, a massive famine affected most parts of China. Causes include collectivization of agriculture and reduction of cultivated land. Estimates of famine-related deaths vary between 15 and 40 million people, making this one of the deadliest tragedies of the twentieth century. The height of the famine differed by region, but in Anhui Province, it was most severe from 1959 to 1960, as indicated by extremely high mortality rates and a reduction in birth rates to one-third of the pre-famine average. A recent study analyzed all in-patient and out-patient psychiatric referrals from 1971 to 2001 to the only psychiatric hospital in the region. The birth cohorts conceived or in early gestation in 1959 and 1960 had a twofold increase in risk for schizophrenia in later life, compared to those who were born before or after the famine (between 1956 and 1958 or between 1963 and 1965) (St Clair et al. 2005). Similarly, in a third study examining the Guanxi autonomous region, another part of China, there was a twofold increase in risk for schizophrenia among those conceived or in early gestation at the height of famine (Xu et al. 2009). The increased risk was found exclusively in rural areas, where famine conditions were most severe. This was due to the provision of food from state grain stores to those living in cities, whereas the rural population was only allowed to retain the grain that remained after they had delivered the imposed quotas.

### *6.2.2 The Folate Pathway*

The biological pathways linking prenatal famine to schizophrenia are currently unknown, but there are many potential mechanisms, including micronutrient deficiencies, maternal stress effects on the neuroendocrine system, toxic effects of maternal ingestion of food substitutes, genetic selection (e.g., genetic differences between women who can and cannot ovulate and conceive during famine conditions), and preconceptional damage to paternal spermatogonia (Brown and Susser 2008).

To illustrate how an environmental exposure may have epigenetic effects in schizophrenia, we introduce the potential mechanism of folate deficiency. It is important to note that the metabolism and functions of folate are complicated (Lucock 2000), and that we use only a simplified example of a pathway in which folate plays a role (Fig. 6.1).

Folate is an important factor in the synthesis and maintenance of DNA, via synthesis of purines and pyrimidines (Lucock 2000). Folate deficiency can lead to chromosomal instability and aberrations of DNA repair, thereby increasing rates of mutation; de novo mutations in multiple genes have been related to schizophrenia (Stefansson et al. 2008). A second potential pathway through which folate deficiency may be related to schizophrenia is by disruption of epigenetic programming; folate is involved in the pathway of DNA methylation (Lucock 2000), which represents one of the major epigenetic processes. Another level of epigenetic information is the methylation of histones, which may similarly affect this process (Feinberg 2007). DNA methylation, meaning the addition of a methyl group to the

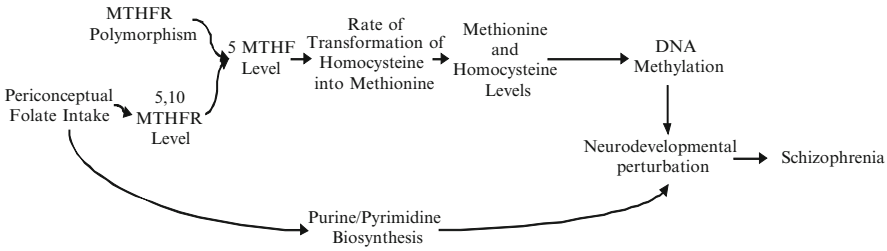


**Fig. 6.1** Pathways that could link prenatal folate deficiency to schizophrenia

nucleotide cytosine, occurs at dinucleotide sites in the DNA where a phosphate molecule links cytosine (C) and guanine (G). The CpG dinucleotides can be found throughout the genome, but are highly concentrated in control regions of genes (CpG islands). Methylation modifies transcriptional access to the DNA and, thus, may alter gene expression (Feinberg 2007). The main source of methyl groups in this reaction is methionine, an essential amino acid that is converted from homocysteine to a biologically active methyl donor state. Folate promotes the conversion to methionine by increasing the level of 5,10-methylenetetrahydrofolate reductase (MTHFR) that in turn catalyzes the conversion of 5,10-MTHF to 5-MTHF, a cosubstrate for the homocysteine remethylation to methionine (Lucock 2000). Theoretically, folate deficiency will lead to a lower level of methionine and, therefore, to a lower rate of DNA methylation (Duthie et al. 2000).

Again, it should be realized that these pathways might be far more complicated than described here. This complexity is illustrated by human studies (including one examining the Dutch famine cohort), which also found associations between low folate status and higher level of DNA methylation in some genes (Tobi et al. 2009; Van Engeland et al. 2003). Also, the putative folate-effect may not only represent damage to the epigenome due to the harsh prenatal environment. It is possible that epigenetic changes in metabolic pathways following exposure to prenatal famine are adaptive, occurring either immediately to survive the malnutrition as an embryo/fetus, or in anticipation of the postnatal environment (Heijmans et al. 2009).

### *6.2.3 Evidence for Influence of Diet on DNA Methylation*

Over the last decade, evidence has accumulated in support of the hypothesis that differences in availability of folate are associated with lasting differences in DNA methylation. There may be changes in epigenetic marks throughout an individual's lifetime (e.g., aging appears to be related to changes in DNA methylation; Rakyan et al. 2010), but the epigenome is the most dynamic in early development. Animal studies have suggested that genome-wide demethylation occurs early in gestation, a process in which most parental epigenetic marks are erased (Reik et al. 2001), with remethylation taking place shortly afterwards. The gestational period in which

epigenetic reprogramming is thought to occur in humans matches the period in which exposure to famine (and thus folate deficiency) was related to an increased risk for neural tube defects and schizophrenia in the Dutch study. Epigenetic effects as a result of folate deficiency may, therefore, be a mechanism that contributes to the increased risk.

Epigenetic marks are determined by genetic and stochastic factors, but as it turns out, they are also under the influence of environmental factors, such as diet, during early development. A series of experiments with agouti viable yellow ($A^{vy}$) mice showed that dietary methyl supplements, including folate, in the early gestational period can produce persistent changes in DNA methylation (Waterland and Jirtle 2003; Wolff et al. 1998). $A^{vy}$ mice have a mutation in the regulatory region of the agouti allele. Ectopic agouti transcription is initiated from a promoter in the proximal end of the inserted transposable element. Methylation in this region varies considerably among individual mice and is correlated inversely with ectopic agouti expression. This epigenetic variability causes a wide variation in individual coat color (Wolff et al. 1998). In the offspring of $A^{vy}$ mice that were assigned to a methyl-rich diet, the agouti gene was methylated to a higher level. This resulted in silencing of the gene and downregulated the ectopic agouti expression, which in turn produced a shift in coat color from yellow to brown (Waterland and Jirtle 2003). Since the color differences persisted into adulthood, these findings demonstrated that early life dietary conditions could cause epigenetic changes that are stable into adulthood.

The Dutch famine study provided a unique opportunity to investigate whether prenatal exposure to the prevailing famine conditions is associated with persistent epigenetic differences in humans. Six decades after World War II, individuals who had been exposed prenatally to famine had less methylation of the imprinted insulin-like growth factor II gene (*IGF2*) than their unexposed same-sex siblings (Heijmans et al. 2008). This association was specific for exposure in early gestation: the group that was exposed in late pregnancy did not have lower *IGF2* methylation than their unexposed siblings, although they did have a lower mean birth weight. A subsequent analysis of 15 genes implicated in growth and metabolic diseases, in the same cohort, found that DNA methylation differences were not restricted to only the IGF2 gene, or to early gestational exposure to famine; some genes had less methylation, other genes had a higher degree of methylation in exposed individuals (Tobi et al. 2009). Methylation differences were sex-specific and some associations were found only in those exposed during late gestation, thus persistent changes in DNA methylation may be a common consequence of prenatal exposure to famine.

### *6.2.4 Epigenetic Effects on Health and the Role of Folate*

Persistent alterations in DNA methylation can have a significant influence on health in animals. This is illustrated by the $A^{vy}$ mice, which not only have a more yellow coat than mice without ectopic agouti expression, but also have increases in obesity,

diabetes, and susceptibility to tumors (Miltenberger et al. 1997; Wolff et al. 1998). However, it should be noted that this is a very specific genetic transposon model and it cannot be generalized to every DNA sequence or genomic element. Most evidence of epigenetic effects on disease in humans comes from cancer research. Tumor development is regulated by the activation of growth-promoting genes through both global and gene-specific hypomethylation or hypermethylation (Feinberg 2007). The methylation differences may be an indication of genomic instability, and also of altered functioning of specific genes that are relevant to specific diseases, for example, silencing of tumor suppression genes through hypermethylation (Feinberg 2007). Other examples include rare epigenetic diseases, such as Beckwith–Wiedemann syndrome, which is characterized by loss of normal imprinted gene regulation (DeBaun et al. 2002), resulting in affected children typically having macrosomia, abdominal birth defects, and an increased risk of childhood cancer.

The finding that early prenatal exposure to famine was related to neural tube defects, combined with nonrandomized trials, small randomized trials, and observational epidemiological studies of maternal vitamin supplements and neural tube defects (Smithells et al. 1980), provided a strong body of evidence that folate vitamin supplements could reduce the risk of neural tube defects, and prompted initiation of large-scale randomized trials. Definitive randomized trials in the 1990s showed that folate supplementation reduced about 80% of the risk for neural tube defects (Czeizel and Dudás 1992; Group MVSR 1991). These studies also suggested that the critical period for the preventive effect of folate supplementation actually begins shortly prior to conception and ends at 28 days of gestation, when the neural tube closes. As a result, from the 1990s onwards, all women who considered pregnancy were advised to take folate supplementation. Some countries even introduced mandatory folic acid fortification of flour, since the benefits of folate supplementation were large, the costs were low and the intervention had no apparent side effects. With increasing knowledge of the folate pathway and of the involvement of folate in epigenetic programming, however, concerns were raised about other potential long-term consequences for child health and development. A recent study found higher methylation of *IGF2* among children whose mother used folic acid during periconception, indicating that this may indeed lead to epigenetic changes in humans (Steegers-Theunissen et al. 2009). The children of the mothers participating in the original randomized trials of folate supplementation have yet to be investigated, however, some observational studies have found associations between maternal use of folate supplements and an increased risk of asthma and atopy in young children (Haberg et al. 2009; Whitrow et al. 2009), while another study has found an association with decreased risk of childhood behavioral problems and hyperactivity (Schlotz et al. 2009). Conversely, one study did not find a relationship between periconceptional folate supplements and mental or psychomotor development (Tamura et al. 2005). Most recently, data from a large prospective Norwegian birth cohort study (Magnus et al. 2006) suggest that folate supplementation is associated with better neurodevelopmental outcomes (results under review). The studies on folate supplementation should all be regarded as

preliminary; nevertheless, they do suggest that periconceptional folate supplements may have consequences for health and neurodevelopment in childhood and later on.

### *6.2.5 Epigenetic Changes in Schizophrenia*

As previously mentioned, epigenetic factors may be related to schizophrenia. Briefly, studies examining postmortem brain tissue reported several potentially important associations. Reelin is a protein involved in neuronal migration in the developing brain, and increased DNA methylation has been found at several positions in the promoter region of the reelin gene in the occipital and prefrontal cortex of schizophrenia patients compared with nonpsychiatric controls (Grayson et al. 2005). A second study, however, showed no detectable DNA methylation at these sites (Tochigi et al. 2008). Another study reported hypomethylation of the membrane-bound catechol-*O*-methyltransferase gene (*MB-COMT*) promoter in the frontal lobe of patients with schizophrenia and bipolar disorder (Abdolmaleky et al. 2006). This may be relevant to the pathophysiology of schizophrenia, since COMT regulates the homeostatic levels of the neurotransmitter dopamine in the synapses, and dopamine plays a central role in schizophrenia (Laruelle 2003). Hypomethylation was related to upregulation of *MB-COMT* (Abdolmaleky et al. 2006), which in turn increases the dopamine degradation in the frontal cortex. *COMT* hypomethylation may be causally related to the cognitive and negative symptoms of schizophrenia, which have been associated with prefrontal hypodopaminergic functioning (Laruelle 2003). Again, a replication study found no evidence of altered *COMT* steady state mRNA or methylation in brains of schizophrenia patients, albeit that this second study used cerebellum rather than frontal lobe tissue (Dempster et al. 2006). In the first genome-wide epigenomic study, Mill et al. (2008) found a number of genes exhibiting differences in DNA methylation in the prefrontal cortex of schizophrenia and bipolar patients vs. healthy controls. The differences included loci involved in glutamatergic and GABAergic neurotransmission and brain development.

There are many caveats in interpreting these findings (see Box 6.1), but even when methodological and conceptual problems are solved, epigenetic studies may continue to yield mixed results when environmental exposures are not taken into account. Given the substantial environmental influence on the epigenome, the epigenetic effects in the pathways leading to schizophrenia may depend on environmental exposures in early life. There are at least two possibilities: (a) environmental exposures may shift the epigenetic status in the exposed population, which results in a higher rate of schizophrenia in that population (Fig. 6.2a) – an example of this principle is that a shift in distribution of high blood pressure in a population can lead to an increase of the occurrence of cardiovascular disease in those individuals (Rose 1985); or (b) epigenetic effects of environmental exposure operate selectively on individuals with genetic predisposition to schizophrenia, i.e., nonadditive gene–environment interaction (Fig. 6.2b).

**Box 6.1. Methodological and Technical Difficulties in Epigenetic Epidemiology**

- Epigenetic marks are cell- and tissue-specific – since the brain cannot be accessed in live individuals, other tissues must be used for epigenetic studies of schizophrenia. Epigenetic marks in tissues such as peripheral blood may not correspond to the epigenome of brain regions.
- The stability of epigenetic marks over the life course is unknown – in cross-sectional studies, it is not possible to test whether epigenetic differences are cause or consequence of illness.
- Interindividual variation in DNA methylation, and the relationships among methylation, gene expression, and functional consequences are not completely clear for most genes.

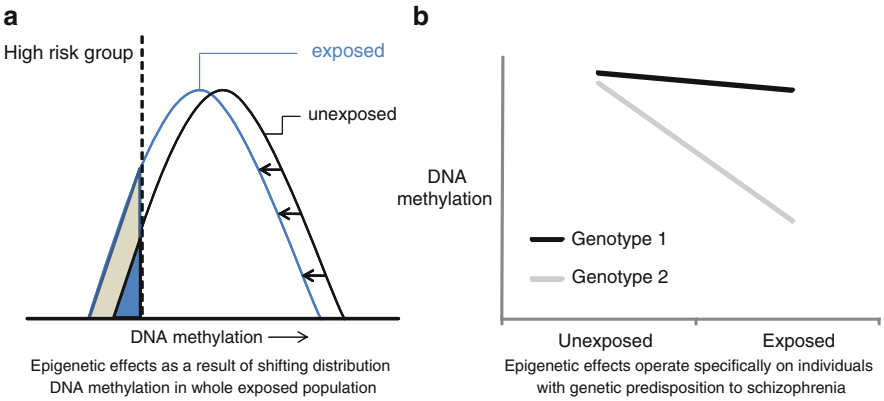


**Fig. 6.2** Possible environmental influences on epigenetic effects and schizophrenia risk

Epidemiological studies can help to detect epigenetic mechanisms of disease, with the conditions that measurement of the environmental exposure is precise, timing of exposure is known, and that a homogeneously exposed population is available for study (Foley et al. 2009; Oh and Petronis 2008). In addition, it only makes sense to study causal epigenetic effects mediated by environmental factors if the correlation between the environmental exposure and genetic predisposition to schizophrenia is low (Oh and Petronis 2008).

### *6.2.6 Famine Studies as a Tool for Detecting Epigenetic Mechanisms in Schizophrenia*

The above conditions can be fulfilled in famine studies. We will use the Dutch famine study for illustration purposes here (Box 6.2). Although the sample size of

**Box 6.2. Is Prenatal Famine a Useful Environmental Exposure for Epigenetic Studies of Schizophrenia?**

The example of the 1944–1945 Dutch famine:

- *Precise measure*: Daily distributed food rations during famine are known. Although some families had more access to food for economic or other reasons, the measures are precise enough to describe changes in average food intake over time.
- *Homogeneous exposure*: The total population of a well-circumscribed geographic area has been exposed to famine, during a well-specified period. While there was substantial individual variation in food intake, the large majority of the population suffered from severe food shortage.
- *Timing of exposure*: Date of birth is an easy measure. It can be used to estimate timing of conception, though with some imprecision.
- *Gene–environment correlation*: War-related famine, it is unlikely that prenatal exposure was strongly influenced by (parental) schizophrenia genes.
- *Plausible biological/genetic pathways*: These are identified; folate-related one-carbon pathway, MTHFR gene, IGF2 gene.

the actual study is too small to investigate epigenetic effects in schizophrenia, there is a great potential for follow-up studies of famine exposure in China, where several hundreds of prenatally exposed schizophrenia cases have been identified (St Clair et al. 2005).

During the World War II famine in the Netherlands, the total population of the larger cities in the western part of the country was exposed to severe food shortages. We know exactly that the height of the famine was from January through April 1945, and that it ended immediately after liberation on 5th May 1945. We know that the daily distributed food ration was less than 500 calories/day at the height of the famine, and we have detailed population data, in addition to detailed information of adult health outcomes, including schizophrenia (Brown and Susser 2008). It is unlikely that a strong correlation exists between (parental) schizophrenia genes and prenatal exposure to famine, because the famine was war-related, unpredicted, and largely inescapable.

In this context, a powerful study design would be to combine the environmental information with epigenetic measures. In a case-control study, DNA methylation should be measured in at least four groups (a) exposed cases – individuals with schizophrenia from the birth cohort conceived during the height of the famine, (b) unexposed cases – individuals with schizophrenia from surrounding birth cohorts, (c) exposed random controls, and (d) unexposed random controls.

Global DNA methylation may be used as an indicator of genomic stability, but hypotheses of gene-specific methylation differences can be tested as well. Methylation loss of the imprinted IGF2 gene is an interesting candidate, because this epigenetic effect has already been found in individuals exposed to the Dutch

famine in early gestation (Heijmans et al. 2008), and the gene is involved in (brain) growth and development. We have to keep in mind that epigenetic marks are tissue-specific, and therefore, the best option would be to study brain tissue, but this cannot be sampled during life. There is some evidence, however, that DNA methylation measured in blood may be a marker for less accessible tissues that are directly involved in disease (Talens et al. 2010). Epigenetic changes in brain tissue are also often found in peripheral cells, but this issue has yet to be resolved.

If epigenetic effects of famine on schizophrenia operate through a shift in distribution of DNA methylation profiles in the total exposed population (Fig. 6.2a), we may find differences in DNA methylation between exposed and unexposed controls (reflecting the shift in distribution), but no differences between exposed and unexposed cases (there will be more exposed than unexposed cases, but in both groups, most cases are found in the high risk end of the distribution).

On the other hand, if epigenetic effects of famine on schizophrenia depend on a specific pre-existing genetic vulnerability to schizophrenia (Fig. 6.2b), we would find a different pattern of results. For example, we may find no differences in DNA methylation between exposed and unexposed controls, but instead find differences between exposed and unexposed cases.

These are two basic illustrations of how epidemiological models can be used and how different models may predict different results. We want to emphasize that many other models can be envisioned, such as a model that accounts for the interplay between the postnatal environment and the epigenome. An example of the latter is discussed in the next section. Nevertheless, the prenatal famine example clearly demonstrates that adding environmental exposure to an epigenetic study may increase the chances of detecting epigenetic effects and understanding the epigenetic mechanisms in schizophrenia. If information about famine exposure status is not considered, the overall result may be inconclusive or not confirmed in replication studies.

## 6.3 Migration/Childhood Ethnic Minority Status

### *6.3.1 Epidemiological Findings*

Associations between the incidence of schizophrenia and international migration have been reported for nearly 80 years. In the early 1930s, Ødegaard (1932) found that Norwegian immigrants in the USA were admitted to a psychiatric hospital for schizophrenia twice as often as native-born Americans or Norwegians in Norway. Observations of higher schizophrenia rates among immigrants were also reported in the 1950s and 1960s in the USA and the UK (Hemsi 1967; Malzberg 1955). These studies had considerable methodological limitations, but later investigations in the UK, using prospective case finding within defined catchment areas, standardized

assessments and diagnostic criteria of psychopathology, and more accurate census data, replicated the findings of an increased risk for schizophrenia in immigrant populations (Harrison et al. 1997; King et al. 1994). In the last decade, the large AESOP incidence study, conducted in London, Nottingham, and Bristol, found strikingly increased rates of schizophrenia in African Caribbeans (Incidence Rate Ratio [IRR] = 9.1; 95% CI 6.6–12.6) and Black Africans (IRR = 5.8; 95% CI 3.9–8.4) compared with the white British population, and modestly increased rates in Asian and other immigrants (Fearon et al. 2006). The rates of schizophrenia were significantly increased in second-generation immigrants in London as well (Coid et al. 2008).

The association between migration and schizophrenia has also been studied extensively in the Netherlands. Selten reported significantly higher first-admission rates for schizophrenia in immigrants from Surinam, the Netherlands Antilles, and Morocco in the 1990s (Selten and Sijben 1994; Selten et al. 1997). Subsequently, a prospective first-contact incidence study of psychotic disorders found an increased risk for schizophrenia in Surinamese and Moroccan immigrants, both in the first and second generation (Selten et al. 2001; Veling et al. 2006). The risk was particularly high for Moroccan males (first generation: IRR = 4.0; 95% CI 2.5–6.3 and second generation: IRR = 5.8; 95% CI 2.9–11.4) and was not significantly increased for Turkish immigrants or immigrants from Western countries (Veling et al. 2006).

Taken together, the incidence of schizophrenia is increased in different immigrant groups in several countries around the world. The degree to which rates are increased varies considerably with ethnic group and country, but the increased risk pertains to second-generation immigrants. A meta-analysis of all migrant incidence studies of schizophrenia up to 2003 estimated the mean relative risks of 2.7 and 4.5 for first- and second-generation immigrants, respectively, compared with native populations (Cantor-Graae and Selten 2005). Several potential explanations have been proposed for the migrant findings. We cannot review these here due to space restrictions (see Cantor-Graae and Selten 2005 for a full discussion), but it is necessary to discuss whether migration can be treated as an environmental risk factor in epigenetic studies of schizophrenia (Box 6.3).

Gene–environment correlation is conceivable, as individuals with genetic predisposition to schizophrenia may be more likely to migrate. No studies in the UK directly addressed selective migration. A population register study in Denmark, however, found an increased risk among immigrants who migrated as a child and, thus, did not make the decision to migrate themselves (Cantor-Graae et al. 2003). In the Netherlands, we recently completed an analysis of age at migration in The Hague incidence study. The results are still under review, but show that the risk for schizophrenia among immigrants is still increased when only those immigrants who migrated before the age of 15 were included.

When age at migration data are available, the relevant timing of exposure can be determined precisely. Our age at migration analyses show that the risk for schizophrenia is particularly high for immigrants who migrated in early childhood (between age 0 and 4) and decreases with older age at migration. In combination with the replicated finding of an increased risk among second-generation immigrants (Coid

**Box 6.3. Is Migration a Useful Environmental Exposure for Epigenetic Studies of Schizophrenia?**

- *Precise measure*: History of international migration.
- *Homogeneous exposure*: First-generation immigrants have all been exposed to migration; the exposure can be considered homogeneous when one country of origin and one country of arrival are studied.
- *Timing of exposure*: Age at migration, easy, and reliable measure.
- *Gene–environment correlation*: Selective migration is unlikely, because the risk for schizophrenia is also increased among immigrants who migrated in childhood.
- *Biological/genetic pathways*: These have not yet been established; hypotheses include sensitization of the mesolimbic dopamine system, and increased inflammatory set-point as a result of stress in early life.

et al. 2008; Veling et al. 2006), the results strongly suggest that not the event of migration itself, but the context of living in an ethnic minority position in early childhood may be the actual environmental exposure influencing risk for psychosis in adulthood.

Several studies in the UK and the Netherlands have found that a disadvantaged ethnic minority position, characterized by low social status, high degree of discrimination against the group, and low proportion of others from one's own ethnic group in the neighborhood (low ethnic density), may lead to an increased risk of psychotic disorders (Veling 2008). This is particularly the case when social resources are insufficient to buffer the impact of the adverse social experiences (compromised family structures, restricted social networks, low access to social capital, and low social cohesion of ethnic group) (Veling 2008). It should be noted that most of these studies investigated the social context at illness onset; however, unpublished data from a US birth cohort study also suggests that low ethnic density at birth increases the risk for adult schizophrenia among ethnic minorities (Dana March, personal communication).

### *6.3.2 Epigenetic Changes in Postnatal Life*

Prenatal folate deficiency can influence epigenetic marks in DNA, such changes may persist into adulthood and may influence health, including neurodevelopmental outcomes. One further step could be to test the association between exposure to famine- and folate-related epigenetic changes in schizophrenia. In the case of migration, however, there are fewer hypotheses of potential pathways and less evidence supporting biological mechanisms. Can we make an argument that epigenetic factors may be involved?

There are two apparent differences, in this respect, between exposure to migration and to famine. First, the epidemiological evidence of migration suggests that early childhood is an important etiological period and, if the effect of migration on schizophrenia is mediated by epigenetic effects, persistent changes in the epigenome should be possible in childhood. This is at least conceivable, as gestation in rats does not equal gestation in humans; brain development in humans takes longer and is still very rapid in the early years of life. Also, the timing of epigenetic programming may be related to tissue differentiation and development, and may occur at a later stage in some tissues (Heijmans et al. 2009). Second, it appears that social context shapes the increased risk among ethnic minorities, rather than dietary conditions. Social experiences should therefore be translated into epigenetic consequences.

Recently, a series of experiments with maternal behavior in rats have suggested that both requirements may be met. Offspring of "high care" mothers are less fearful and show a more modest physiological stress response than offspring of "low care" mothers (Caldji et al. 1998), but biological offspring of "low care" mothers reared by "high care" mothers (cross-fostering) resemble the normal offspring of the "high care" mothers (Francis et al. 1999). These findings suggest a persistent effect of maternal behavior on stress reactivity in offspring that is not associated with DNA sequence. The glucocorticoid receptor (GR) may be involved, because GR gene expression is related to stress reactivity and was increased in the offspring of "high care" mothers (Francis et al. 1999). As gene expression is controlled by the epigenome, Meaney and colleagues investigated whether maternal care alters the methylation of the promoter region of the GR gene in rats (Weaver et al. 2004). They found de novo differences in methylation of the NGFI-A consensus sequence on the exon $1_7$ promoter in hippocampal cells between offspring of low care and high care mothers (Weaver et al. 2004). These differences were absent at birth, but emerged over the first week of life and persisted into adulthood. The impact of maternal care on DNA methylation was reversible in adult postmitotic brain cells, as differences in DNA methylation were removed after central infusion of a histone deacetylase (HDAC) inhibitor (Weaver et al. 2004). In another experiment, both methylation pattern and behavioral response to stress normalized after methionine treatment in adult offspring of "low care" mothers (Weaver et al. 2005).

Animal studies also suggest that epigenetic mechanisms are involved in the formation of long-term memory in the hippocampus. Neurons of rats subjected to a conditioned fear paradigm (in which they learn to associate a novel context with an aversive stimulus) showed increased acetylation of histone H3 (Levenson et al. 2004). DNA methylation was also found to regulate synaptic plasticity and memory in adult animals, while fear conditioning was associated with rapid methylation of the memory suppressor gene protein, phosphatase 1, and demethylation of the reelin gene (Miller et al. 2008; Sweatt 2009).

These results suggest that epigenetic regulation of genes may be a much more dynamic process than previously believed. Environmental influences on the epigenome might not be restricted to prenatal life – early social experiences, such as maternal care, may cause epigenetic differences with adult phenotypic consequences. Also, despite the inherent stability of epigenomic marks established in

early life, they may be potentially reversible in the adult brain. Moreover, epigenetic processes may be involved in normal brain responses to environmental stimuli.

It should be noted, however, that it is uncertain how these rat models apply to human physiology and psychology. We do not know whether social experiences affect epigenetic programming in humans. It is also unclear whether significant epigenetic changes due to environmental exposures occur throughout life or are limited to critical points perinatally and perhaps in early life (Szyf et al. 2008). Still, there is some evidence of changes in epigenetic patterns over the life course: one report suggests that monozygotic twins may show increasingly different global DNA methylation profiles at older ages (Fraga et al. 2005), and a recent study of normal human aging identified aging-associated differentially methylated regions in different tissues (Rakyan et al. 2010). These changes may reflect not only the genetic control, but also the influence of environmental exposures over the life course. One recent study examining epigenetic regulation of the glucocorticoid receptor gene (*GR*) in the human brain suggests that the maternal care results in rats may be relevant to humans. Suicide victims with a history of childhood abuse were found to have decreased levels of *GR* mRNA, as well as mRNA transcripts bearing the *GR 1F* splice variant and an increase in cytosine methylation of the *NR3C1* promoter, compared to either suicide victims with no childhood abuse or controls (McGowan et al. 2009).

These issues have not been resolved as of yet, and epidemiological studies are needed to clarify the role of epigenetics over the life course. Migration studies can help to test whether social experiences of early life adversity can have long-term effects on epigenetic processes in humans, and whether and how these may increase the risk of schizophrenia.

### *6.3.3 Ethnic Minority Status and Potential Epigenetic Effects*

As previously discussed, the high rates of schizophrenia among ethnic minorities are difficult to explain outside of the context of social factors. More specifically, having an ethnic minority status in early childhood is related to an increased risk. Few biological hypotheses have been proposed to explain these findings, and none have been tested in humans. We use two examples to illustrate potential epigenetic pathways.

Social defeat has been used as a paradigm to relate epidemiological findings to potential neurobiological mechanisms. Selten and Cantor-Graae (2007) proposed that childhood ethnic minority status represents the long-term experience of social defeat, defined as a subordinate position or as "outsider status." In rodents, chronic exposure to social defeat (carried out by placing an experimental mouse in the home cage of a different aggressive mouse repeatedly for several days) has been associated with dopaminergic hyperactivity in the mesocorticolimbic system (Tidey and Miczek 1996), in particular, when the experimental animals were socially isolated after defeat (Isovich et al. 2001). Heightened dopaminergic transmission is likely to play a central role in schizophrenia. Several studies demonstrated that drug naïve

schizophrenia patients have an increase in mesolimbic dopamine release after acute amphetamine challenge (Laruelle 2003), suggesting an abnormal responsiveness of dopaminergic neurons. Among other factors, the neurotrophic factor brain-derived neurotrophic factor (BDNF) may be a key regulator of the mesolimbic dopamine pathway (Berton et al. 2006). In an animal study, knockdown of *Bdnf* in the nucleus accumbens (NAc) removed most of the effects of repeated social defeat on gene expression within this circuit (Berton et al. 2006). Another study reported that chronic social defeat stress significantly downregulated mRNA levels of HDAC 5 in the NAc (Renthal et al. 2007). This suggests epigenetic control of the response to social defeat, as this HDAC enzyme is capable of repressing *BDNF*, as well as the expression of many other genes (Tsankova et al. 2006).

The second example involves immunological pathways. The hypothesis that chronic inflammation may be a basic biological mechanism of schizophrenia has a long history (Smith 1992). Recent studies of the proinflammatory state of circulating monocytes in patients with bipolar disorder and schizophrenia have reported increased mRNA levels of inflammatory genes (Drexhage et al. 2010; Padmos et al. 2008). The aberrant gene-expression profile was also found in children of bipolar patients (Padmos et al. 2008), and a subsequent twin study suggested that nearly all the variation in proinflammatory gene expression was due to environmental factors (Padmos et al. 2009). There are a multitude of factors that can influence proinflammatory monocyte activation, one of which is exposure to stress in early life (Padmos et al. 2009). Growing up in a low ethnic density context, which is related to increased risk for schizophrenia among ethnic minorities (Veling et al. 2008), is likely to be associated with disturbing experiences and social stress – stress is often implicated in etiological models of schizophrenia (Walker and Diforio 1997). Consistent with this hypothesis, human studies have found that individuals who are genetically vulnerable to psychosis are also more sensitive to daily life stress than healthy controls (Myin-Germeys et al. 2001, 2005). In addition, early childhood trauma has been linked to an increased risk for adult psychosis (Read et al. 2005), and there is preliminary evidence that early trauma may have persistent epigenetic effects on stress reactivity (McGowan et al. 2009). Overall, the maternal care experiments in rats discussed earlier suggest the possibility of epigenetic regulation of stress reactivity (Weaver et al. 2004), and evidence in humans supports that there is an inherent plausibility that different, disturbing experiences in early childhood could have epigenetic effects. In summation, the epigenetic modifications induced by early life stress exposure may mediate the effect of childhood ethnic minority status on schizophrenia.

### *6.3.4 Migration Studies as a Tool for Detecting Epigenetic Mechanisms in Schizophrenia*

Migration studies provide opportunities to investigate potential epigenetic mechanisms in schizophrenia. To enhance the power of the study design and to increase the

homogeneity of the exposure, we should study a single ethnic minority group, preferably the group with the highest effect of migration on risk for schizophrenia, i.e., African Caribbeans in the UK or Moroccans in the Netherlands.

The first question to address is whether early postnatal social experiences can influence the epigenome in humans. That is are persistent epigenetic changes associated with early postnatal exposure to the social experience of ethnic minority status? To test this concept, we could assess peripheral blood samples and compare the epigenome of exposed ethnic minority individuals (who arrived in the Netherlands before age 5 or were born in the Netherlands as second generation) with that of unexposed individuals (ethnic minorities who arrived as adults or are native Dutch). Again, an important caveat is that it is uncertain how epigenetic marks in peripheral blood correspond to the epigenome of the brain tissues relevant to schizophrenia. Epigenetic changes occurring later in life in one tissue will not necessarily be visible in another tissue, whereas epigenetic changes induced early in development may be more likely to be propagated throughout the body because the epigenetic marks are partially stable in somatic cells (Heijmans et al. 2009).

The second question is whether: epigenetic changes mediate the effect of ethnic minority status on the risk for schizophrenia. As explained in the famine example, epigenetic effects of childhood ethnic minority status may shift the whole distribution of epigenetic changes in the ethnic minority population (Fig. 6.2a), or they may operate selectively on individuals with a genetic predisposition to schizophrenia (Fig. 6.2b). If epigenetic effects operate through a shift in distribution of DNA methylation, we would find differences in DNA methylation between exposed and unexposed controls (reflecting the shift in distribution), but no differences between exposed and unexposed cases (there will be more exposed than unexposed cases, but in both groups, most cases are found in the high risk end of the distribution). However, if epigenetic effects of childhood ethnic minority status depend on specific pre-existing genetic vulnerability to schizophrenia (Fig. 6.2a), we may find differences in DNA methylation between exposed cases and exposed controls, but no significant epigenetic differences between unexposed cases and unexposed controls.

## 6.4 Conclusion

Epigenetic mechanisms may play an important role in the etiological pathways of schizophrenia. Since the epigenetic status of the genome partly depends on environmental factors in pre- and postnatal environments, exposure to such factors should be taken into account in epigenetic studies of schizophrenia. Prenatal famine and childhood ethnic minority status have been identified as environmental risk factors for schizophrenia. These exposures can be used to investigate epigenetic effects on schizophrenia, since environmental exposure can be measured with sufficient precision, homogeneously exposed populations are available for study, and plausible biological pathways have been suggested (albeit less specific for

migration). We have shown that epidemiological studies of famine and migration can help to detect epigenetic mechanisms in schizophrenia, by comparing the epigenome of exposed and unexposed schizophrenia cases and controls. Since epigenetic marks are tissue-specific, especially when they occur later in life, experimenters should carefully consider the choice of tissues to be used in these studies. The results of the comparisons will be different depending on the mechanism involved in the interplay between environment and epigenome. If these epidemiological designs are not applied, the overall result of epigenetic schizophrenia studies may well continue to be inconclusive. Thus, the examples of prenatal famine and childhood ethnic minority status clearly demonstrate that it is important to add an environmental exposure aspect to epigenetic studies of schizophrenia.

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# Chapter 7
# Imprinting, Inactivation and the Behavioural Genetics of the X Chromosome

**Ian W. Craig**

**Abstract** The X chromosome presents some unique features in the context of DNA modifications including methylation and histone deposition. In some ways, the patterns of epigenetic changes during the life cycle of the X chromosome resemble those affecting autosomal loci in parent-of-origin effects. Similarly, chromatin changes to the X chromosome of somatic cells may occur in response to the impact of external environmental factors. In addition to any imprinting, the X chromosome of placental mammals has the distinction of random inactivation in diploid females. The focus of this chapter is to examine what consequences these X-chromosomal modifications may have for human phenotypes and in particular that of behaviour. It is of particular interest in this context that, of the few loci escaping inactivation and expressed at higher levels in females, several are notable for their involvement in chromatin remodelling.

**Keywords** Behaviour · Chromatin remodelling · Histone modification · Twin studies · X-inactivation

## 7.1 X Chromosome Imprinting and Inactivation Cycle

As reviewed elsewhere in this volume, one of the most easily studied aspects of epigenetic modification is that of methylation of the cytosine residue at CpG dinucleotides. Studies in mice indicate that, at fertilisation, about 20% of CpG sites are methylated. At the blastocyst stage, there is a dramatic removal of most of these (although it is supposed that a number of key methylated cytosine marks may be maintained) to be later followed by waves of de novo methylation at the trophoblast stage – with somatic and germ cell lineages subsequently achieving different overall levels of about 60 and 30%, respectively (Reik et al. 2001;

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_7,
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Jaenisch and Bird 2003). Following fertilisation, there are significant differences in the mechanisms involved in the demethylation of maternal and paternal genomes. Whereas the chromatin of paternal chromosomes is reprogrammed before replication by an active process, which involves the replacement of protamines by acetylated histones and extensive demethylation, loss of methylation from the maternal genome is replication dependent (Reik et al. 2001).

Against this genomic background, subsequent epigenetic modification of the X chromosome also follows different pathways depending on the parent of origin. The paternal X ($X^p$) arrives in the zygote in a pre-inactivated state because, during the meiotic process in spermatogenesis, its regions that do not pair with homologous regions on the Y undergo inactivation in a process that appears to differ mechanistically from that seen in the differentiating embryo and which does not involve the *Xist* locus (see below). Furthermore, it seems that following meiotic inactivation in males, some regions of the X are reactivated in spermatids enabling many multi-copy genes, including some encoding testes-specific transcripts, to be expressed. Following fertilisation, preferential inactivation of $X^p$ is followed by its reactivation and the subsequent random inactivation of maternally and paternally inherited X chromosomes. Unlike spermatogenic meiotic inactivation, the pathway of preferential paternal X-inactivation that follows fertilisation appears to require de novo *Xist* expression (Mueller et al. 2008).

### *7.1.1 The Role of XIST in X Chromosome Epigenetic Modification*

Initiation of random X-inactivation, which is the norm for somatic tissues of eutherian mammals, requires both that the number of X chromosomes relative to autosomes be counted and that a random choice made as to which of two Xs in a diploid chromosome set is inactivated (Heard and Disteche 2006). The locus controlling this process is referred to as the inactivation centre (*XIC*). This region (located at Xp13.2 in humans) contains several elements and regulatory sequences. Much of what is known concerning X-inactivation in eutherian mammals is based on studies in mice with some support from parallel investigations in humans. Although there are some differences in precise mechanism, it is recognised that one common aspect of epigenetic modification is the role of the product of the *XIST* locus (referred to as *Xist* in mice) located within the X-inactivation centre (*XIC* – *Xic* in mice). *XIST* is responsible for the production of the non-translated RNA that coats and interacts with the chromosome and which signals the first stage of inactivation (Ng et al. 2007). This precedes histone modifications and the extensive methylation of CpG dinucleotides that together are responsible for the relatively stable subsequent silencing of a majority of the X chromosome's transcriptional activity.

In mice, at the first examinable stages following fertilisation, both paternal and maternal X chromosomes appear to be active and both *Xic* sites switched off

(Okamoto and Heard 2006); however, there persists some epigenetic mark on the paternal X predisposing subsequent preferential *Xist*-linked inactivation. Such imprinting-linked inactivation of the paternal X persists in all tissues of marsupials and in the extra-embryonic membranes of some eutherians including mice and to a limited degree in human trophoblasts. The paternal X-inactivation is well established by the blastocyst stage and some studies suggest its onset as early as the eight-cell stage (Heard and Disteche 2006). This $X^p$-specific inactivation is generally accompanied by similar chromatin changes to those seen in regular inactivation observed in cellular differentiation, although CpG methylation of gene promoters does not feature. Murine paternal X-inactivation is consolidated in trophoectoderm; however, in the embryo proper (inner cell mass), the inactive $X^p$ become reactivated during blastocyst differentiation, coincident with a general erasure of epigenetic marks throughout the genome at this point. This, thereby, sets the stage for the process of random inactivation of paternal and maternal chromosomes – mediated by a counting process that results in the inactivation of $N - 1$ X chromosomes, where $N$ is the total number of Xs. Hence in XXX individuals only a single one remains active. The condensed (and hence inactive X) chromosome in females was first described by Barr and Bertram (1949) and as a result has subsequently been referred to as a Barr body. Although some details differ, specifically with respect to a key role in mice for an antisense transcript to *Xist* (*Tsix*), overall murine and human patterns appear to be similar.

## 7.2 Skewing of X Chromosome Inactivation

Broadly speaking, X-inactivation enables males and females to achieve parity in gene expression; however, as a result of the contribution of genes that escape inactivation, potentially important consequences arise for sex differences in behaviour. Additional complications may arise as a result of skewing in the inactivation process. It is of interest that, although it might be anticipated that random inactivation in somatic tissues should result in a 50:50 distribution between cells having an active paternal X ($X_p^a$) and those having an active maternal X ($X_m^a$), quite significant differences in inactivation ratios are observed in practice. These can result from a variety of genetic and stochastic processes. For example, although inactivation in differentiating cells is generally independent of parental origin, in mice, different strains carry X-inactivation centres of differing "strengths" so that in inter-strain crosses the X from one strain may preferentially remain active. This is thought to be controlled by a locus-controlling variation and which is coincident with a regulator of *Tsix*, the locus encoding an antisense transcript to *Xist* (Ogawa and Lee 2003; Boumil et al. 2006). There is also evidence in humans that, in a possibly analogous manner, skewed X-chromosome inactivation segregates in some pedigrees in a manner consistent with mutations affecting the *XIST* locus (Plenge et al. 1997). In other cases, extremely skewed inactivation patterns may arise from X-chromosomal mutations that may result in

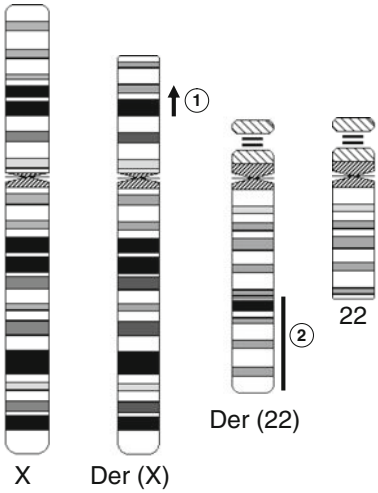


**Fig. 7.1** Chromosome ideograms illustrating an X:autosome translocation (in this case X;22) and the consequences arising from inactivation. If the normal (non-rearranged) X is inactivated, any gene that would normally be expressed, but which crosses the breakpoint, will be rendered non-functional and consequently, the cell will lack any product from either X-locus. If the rearranged (der X) is inactivated, two consequences arise. Firstly, the inactivation spread from the *XIST* locus may penetrate into the portion of chromosome 22 attached. Secondly, the distal part of the X short arm will fail to be inactivated. The latter result particularly is likely to have cell-lethal consequences leaving only the cells with an inactive normal X to survive and hence the female translocation carrier will manifest any pathological symptoms associated with the lack of product from any gene situated across the breakpoint. In the case illustrated, the translocation carrier would exhibit Duchenne muscular dystrophy, as the DMD gene is located at Xp21.2. Ideograms drawn using http://www.cydas.org/OnlineAnalysis/ (Hiller B, Bradtke J, Balz H and Rieder H, 2004)

selection against cells carrying the defective X (Heard et al. 1997). X chromosome translocations also often result in selective cell death as a result of isolation of X-chromosomal regions from the inactivation centre, which can result in diploid expression of normally inactivated X-linked genes, as is the case in girls with X autosomal translocations. Surviving cells will have an inactive normal X and will lack expression of any gene crossing the breakpoint on the derived X chromosome with potentially pathological consequences (Fig. 7.1). Finally, skewing may simply result from stochastic processes, particularly if tissues develop from relatively few cells and the number of stem cells existing at the various stages will affect any skewing observed. This process is generally thought to underpin most of the deviations from a 50:50 distribution of inactive $X^{p}$ and $X^{m}$ observed. Indeed, skewing in favour of the activity of one or other of the X chromosomes is relatively common in adults, with about 35% of individuals showing $>$ 70:30 skewed in either direction and with severe skewing (ratios $>$ 90:1) seen in 7% of women under 25 and in 16% of women over 60 (Sharp et al. 2000; Amos-Landgraf et al. 2006), probably as a result of random events occurring on a small number of progenitor cells.

The X chromosome skewing resulting from X mutations and translocations can have very significant phenotypic consequences. Skewed inactivation in the absence of obvious chromosomal aberrations can also cause profound phenotypic differences even between otherwise genetically identical individuals, most convincingly evidenced by monozygous (MZ) girl twin pairs discordant for the expression of haemophilia, fragile X mental retardation and Duchenne muscular dystrophy (Tiberio 1994; Brown and Robinson 2000). The significance of skewed inactivation leading to the identification of X-linked quantitative trait loci (QTLs) affecting behaviour through twin studies is discussed later.

## 7.3 Chromatin Modifications Associated with the Inactive X Chromosome

In general, active regions of chromosomes are packed in a loose structure (euchromatin) that contains a high proportion of histone H3 and H4 molecules with acetylated lysine residues at their N-terminal tails and, where present, active genes are characterised by unmethylated CpG islands; furthermore, the promoter regions of active genes are frequently marked by methylation of lysine-4 of histone H3 (H3-K4). In contrast, heterochromatic regions reflect an inactive status and, in addition to the methylation of CpG islands, are typically enriched in methylated lysine residue 9 at the terminus of histone 3 (H3K9me), a modification that is observed from early stages of X-inactivation (Heard et al. 2001). The categorisation into either euchromatic or heterochromatic state, however, does not depend solely on a particular epigenetic mark, but on the collective influence of many different modifications. The inactivation of the X chromosome in differentiating cells can be separated into two distinct stages. The first involves the initiation and primary steps in which *XIST* RNA plays a key role, although this may be dependent on the particular cellular context (Sengupta et al. 2008). The complete manifestation of inactivation requires a regulated multi-layering of chromatin modifications. Transition from initiation to maintenance phase appears to involve the polycomb group proteins – known for their activity in repression of the *Hox* genes and which are implicated in the control of body patterns and cell differentiation. Other chromatin and nuclear architectural proteins are recruited to enforce the inactivation status; these include scaffold attachment factors, the trithorax group protein Ash2l and the histone variant macro-H2A2.1 (Pullirsch et al. 2010). As in other chromatin silencing, X-inactivation is typically characterised by additional changes in histone methylation, histone deacetylation and DNA methylation. Once stable inactivation is established, the X chromatin can be divorced from its *cis*-acting inactivation centre and its epigenetic downregulation will persist in its complete absence.

## 7.4 The Spread of X-Inactivation and the Role of LINE Elements

It has long been recognised that the spread of inactivation and the epigenetic marks that accompany it do not penetrate far into the autosomal sequences adjacent to the X chromosome in X/A translocations and it has been therefore surmised that changes in the pattern of genomic features may be responsible for the lack of transmission. Mary Lyon (1998) suggested that LINE-1 motifs may be the key elements forming the way-stations that had been proposed to exist along the spreading inactivation path (Gartler and Riggs 1983). The LINE-1 hypothesis is circumstantially supported by their approximately twofold higher density on the X chromosome sequence (29%) compared to only 17% for autosomes and their relative dearth around loci for which evidence exists that they escape the inactivation process. LINE-1 motifs are particularly enriched around the inactivation centre as predicted for agents instrumental in propagating the inactivation process (Ross et al. 2005). Further support for the model comes from studies on an X/4 translocation in a female mouse embryonic stem cell line, T37H. Popova et al. (2006) demonstrated that the ingress of inactivation into chromosome 4 material, as evidenced from the spreading of *Xist* RNA and histone hypoacetylation, from the *Xic* locus was correlated with its encounter with an extensive 20 Mb region depleted in Line-1 sequences located close to the translocation breakpoint. Other features may also control the spread of inactivation; for example, studies on genes believed to escape inactivation revealed that their 5′ ends were protected by CTCF insulator elements (Filippova et al. 2005).

## 7.5 Patterns of Inactivation and Loci Which Escape

In the foregoing account, it has been assumed that genes on the inactive X are not expressed, apart from regions of X/Y homology that include both pseudoautosomal regions and some scattered isolated regions mostly found on the X short arm in humans. Obviously, transcripts from any loci that escape inactivation and which do not have expressed Y homologues may be expected to be found at relatively higher concentration in females than males and potentially contribute to sex differences in phenotypes including behaviour (Craig et al. 2004a). Because of this, there has been considerable interest in establishing the number and nature of any such escapee genes.

A variety of strategies have been employed to track the potential expression and hence the inactivation status of specific loci on paternal and maternal X chromosomes and resulted in widely differing conclusions. Early studies were based on animals and human individuals heterozygous for the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD), which was taken as a representative marker for the inactivation status of the entire X. Heterozygotes were chosen to

have electrophoretically distinguishable allelic variants. If both alleles are active in cells or tissues, an intermediate hybrid dimeric band appears following electrophoresis of the enzyme resulting in a three-banded pattern. In contrast, single cells or clonal cell lines with $X^p$ or $X^m$ exclusively active exhibit a single band, and tissues with a mixture of both cell types will exhibit two bands with no intermediate form. The presence of a hybrid-band pattern in post-meiotic germ cells in both humans and mice constituted evidence for X-chromosome reactivation during female germline development (Gartler and Riggs 1983). This inactivation assay was later employed in studies leading to the first strong evidence that at least one human X-linked gene (steroid sulphatase, STS) escapes partially from inactivation (Gartler and Riggs 1983). Since this time, a variety of other approaches has been employed to monitor the inactivation status of X-linked genes and to assess the degree of skewing, thereby enabling an investigation of the relationship between CpG island methylation, transcription and mono-allelic expression. Many methods employed to monitor inactivation patterns have been based on the epigenetic methylation of CpG islands associated with genes on the inactive X. This results in resistance to digestion by one of the isoschizomer pairs of restriction enzymes, for example *Msp*I and *Hpa*II, both of which cleave at CCGG sites; however, digestion with *Hpa*II but not *Msp*I is inhibited by methylation of the internal cytosine. The size of products from PCR amplification across the non-digested template of heterozygous females can then be distinguished by the selection of target regions that contain both the CCGG site(s) and appropriate variable tandem repeat polymorphisms allowing the products to be distinguished. Systems based on tandem repeats at the androgen receptor, AR (Allen et al. 1992), and the DXS255 loci have been commonly employed (Boyd and Fraser 1990).

More recently, the focus has shifted to studies scanning the entire chromosome for loci and regions that remain active on an otherwise inactive X chromosome. One of the first preliminary chromosome-wide studies was based on the application of non-quantitative PCR detection of human transcripts from somatic cell hybrids carrying an inactive human X chromosome in an essentially otherwise rodent cell-line background. It was concluded that about 20% of genes tested escaped inactivation and that a significant proportion of these were located on the short arm (Carrel et al. 1999). This study was followed up with more detailed investigations employing a similar strategy supplemented with a quantitative allele-specific assay on a subset of loci examined. Broadly similar general conclusions were reached with about 15% of loci escaping to some degree with an additional 10% of X-linked genes showing variable patterns and extents of inactivation (Carrel and Willard 2005). The somatic cell hybrid approach, however, will detect even low levels of expression from an inactive X and furthermore relates specifically to the situation in a transformed rodent cell background, which may not represent the environment of a more typical somatic cell. Other techniques such as quantitative evaluation of data from expression microarrays are better suited to assess whether, or not, escapees contribute overall to differences in gene dosage between males and females. Comparisons between cell lines with supernumerary X chromosomes, and on lymphocytes,

lymphoblastoid lines and tissues from males and females have been reported (Sudbrak et al. 2001; Craig et al. 2004b; Talebizadeh et al. 2006; McRae et al. 2007). In a comprehensive survey, Johnston et al. (2008) established that male and female expression levels were generally similar and, in an important extension to the observations of Nugyen and Disteche (2006), noted that expression from the single X in male cell lines is upregulated twofold relative to autosomes, thereby achieving dosage parity between the X and autosomes. In addition, they provided compelling data that relatively few loci consistently escape inactivation in females.

Confidence in the potentially significant implications of the microarray expression approach is the coincident identification of some key regulatory and protein-modifying loci in several of the studies. These are highlighted in bold in Table 7.1. Of particular relevance in the present context of epigenetics and disease is the identification as escapees of *SMC1L1* (HGNC Symbol: *SMC1A* – structural maintenance of chromosomes 1A) and of its close distal neighbour *SMCX*. SMC1L1 is a component of the cohesion complex, which, in turn, interacts with PDS5, a protein implicated in chromosome cohesion, condensation and recombination in yeast. *SMCX* encodes one of the families of histone H3 lysine-4 demethylases and reverses the trimethylated H3K4me3 to di- and mono-methylated status and presumptively results in transcriptional repression at target sites. *SMCX* is also one of the more frequently mutated targets in X-linked mental retardation (XLMR) and there is evidence that it both escapes inactivation and has a distinct function from its Y-linked counterpart (*SMCY*). In a manner similar to some of the other confirmed loci that escape inactivation, its Y homologue differs in amino acid sequence and in its levels and patterns of expression. Both *SMCX* and *SMCY* are expressed in mouse brain in a sex-specific fashion with *SMCX* being expressed at a significantly higher level in the adult mouse brain of females compared with males, and expression of the Y homologue in males being insufficient to compensate for the female bias in X-gene expression (Xu et al. 2002). Moreover, *SMCX* has been implicated in neuronal survival and dendritic survival and its mutation in several XLMR patients is associated with reduced demethylase activity (Iwase et al. 2007).

UTX (ubiquitously transcribed TPR gene on the X chromosome) is another gene escaping inactivation and which is involved in epigenetic modification. It catalyses the demethylation of tri/di-methylated H3 lysine-27 (H5K27me3/2) and has been implicated in HOX gene regulation. A further escapee with potentially important significance in epigenetic regulation is *MSL3L1* (male-specific lethal 3-like 1) – given that *Drosophila* male-specific lethal (msl) genes regulate transcription from the male X chromosome in a dosage compensation pathway (see http://www.ncbi.nlm.nih.gov/omim/ for details).

It is, therefore, of considerable interest to discover that, of the relatively few genes that escape inactivation, several have roles in epigenetic modification and consequently have considerable potential for gene regulation. Overall, it is reasonable to suppose that such loci have the potential to contribute to sex differences in a range of phenotypes including behaviour.

**Table 7.1** Loci escaping inactivation based on over-expression in females detected by microarray analyses

| Gene name | Escapees Johnston et al. (2008) | Escapees Craig et al. (2004b) | Escapees Talebizadeh et al. (2006) | Escapees McRae et al. (2007) | Escapees based on somatic cell hybrids Carrel and Willard (2005) | Position Kb from pter |
|---|---|---|---|---|---|---|
| *ACSL4* | | | Yes | | 0 of 5 | 108,884,564–108,976,621 reverse strand |
| *ALG13* | Yes | | | | | 110,909,043–111,003,877 forward strand |
| *AP1S2* | | | Yes | | 9 of 9 | 15,843,929–15,873,100 reverse strand |
| ***DDX3*** | | Yes | | Yes | 9 of 9 | 41,192,651–41,223,725 forward strand |
| ***CA5B/CA5BL*** | Yes | | | Yes | | 15,706,953–15,805,747 forward strand |
| *CDR1* | | | Yes | | 2 of 9 | 139,865,425–139,866,723 reverse strand |
| *CLCN4* | | Yes | | | 5 of 9 | 10,125,024–10,205,700 forward strand |
| ***EIF1A*** | Yes | | | Yes | | 20,142,636–20,159,962 reverse strand |
| ***EIF2S*** | Yes | Yes | | Yes | 9 of 9 | 24,072,833–24,096,088 forward strand |
| ***EIF2S3*** | | | | | | |
| *FUNDC1* | Yes | | | | | 44,382,885–44,402,247 reverse strand |
| ***HDHD1A*** | Yes | | Yes | Yes | 8 of 9 | 6,966,961–7,066,231 reverse strand |
| ***MSL3L1/MSL3*** | Yes | | | Yes | | 11,776,278–11,793,870 forward strand |
| ***PCTK1*** | Yes | Yes | | | 9 of 9 | 47,077,528–47,089,396 forward strand |
| *RBBP7* | | | | Yes | | 16,857,406–16,888,537 reverse strand |
| ***PNPLA4*** | Yes | | Yes | | 9 of 9 | 7,866,288–7,895,780 reverse strand |
| ***PRKX*** | Yes | | | Yes | | 3,522,411–3,631,649 reverse strand |

(*continued*)

**Table 7.1** (continued)

| Gene name | Escapees Johnston et al. (2008) | Escapees Craig et al. (2004b) | Escapees Talebizadeh et al. (2006) | Escapees McRae et al. (2007) | Escapees based on somatic cell hybrids Carrel and Willard (2005) | Position Kb from pter |
|---|---|---|---|---|---|---|
| *RPGR* | | | Yes | | 0 of 9 | 38,128,424–38,186,817 reverse strand |
| ***RPS4X*** | Yes | | Yes | | | 71,475,529–71,497,150 reverse strand |
| *SEDL/TRAPPC2* | | Yes | | | 9 of 9 | 13,730,363–13,752,742 reverse strand |
| ***SMC1L1/SMC1A*** | Yes | Yes | | Yes | 7 of 9 | 53,401,070–53,449,677 reverse strand |
| ***SMCX/JARID1C*** | Yes | | | Yes | | 53,221,334–53,254,604 reverse strand |
| *SRSP2/SMS* | | Yes | | | 9 of 9 | 21,958,691–22,025,318 forward strand |
| *STK3* | | Yes | | | 9 of 9 | 131,157,245–131,209,971 forward strand |
| ***STS*** | Yes | Yes | | | 9 of 9 | 7,137,497–7,272,851 forward strand |
| *SYP* | | Yes | | | 2 of 9 | 49,044,269–49,056,718 reverse strand |
| *TBL1* | | Yes | | | 7 of 9 | 9,431,335–9,687,780 forward strand |
| ***U2AF1L2 ZRSR*** | Yes | | | Yes | | 15,808,595–15,841,383 forward strand |
| ***UBE1/UBA1*** | Yes | Yes[a] | | | | 47,050,260–47,074,527 forward strand |
| *USP9X* | Yes | | | | | 40,944,888–41,092,185 forward strand |
| ***UTX/KDM6A*** | Yes | Yes | Yes | Yes | | 44,732,423–44,971,847 forward strand |
| ***ZFX*** | Yes | Yes | Yes | Yes | 9 of 9 | 24,167,290–24,234,206 forward strand |

[a]Weak signal and very partial escape

## 7.6 Somatic Imprinting of X-Linked Genes and Potential Impact on Behaviour

The chromosome constitution of individuals with Turner's syndrome (TS), who have a single X and no other sex chromosome (45, X0), provides an opportunity to examine possible differences in expression from paternally or maternally inherited X chromosomes. In a series of studies on Turner's syndrome females, a range of behavioural phenotypes were observed, which appeared to differ depending on the parent of origin of the single X. Those who retained the maternal X ($X^m0$) had greater difficulties in socialisation than those with a paternal X (Skuse et al. 1997). The authors speculated that an X-linked gene (or group of genes) existed, which was implicated in social behaviour and which was inactive (imprinted) on the maternally inherited X chromosome. This raised the interesting possibility that the generally accepted relative lack of such skills in boys is a result of their inheritance of their X chromosome from their mothers. Other evidence for X-linked socialisation QTLs has come more recently from twin studies (see below). A variety of subsequent investigations has supported the existence of behavioural differences in $X^p0$ compared with $X^m0$ Turner's syndrome individuals; however, the situation is complicated by the existence of mosaicism where some individuals may retain some tissues containing cells with additional sex chromosome fragments (Henn and Zang 1997). Although there is some direct experimental evidence for imprinted loci in mice (Davies et al. 2005; Raefski and O'Neill 2005), none is yet available for humans and the issue requires further clarification.

## 7.7 Behavioural Impact of Epigenetic Status at Specific X-Linked Genes

### *7.7.1 Fragile X Mental Retardation Syndrome, FRAXA*

Fragile X syndrome is the most common inherited cause of mental retardation, with an incidence of around 1 in 4,000 males (Turner et al. 1996). The mental impairment is often co-morbid with further symptoms including macro-orchidism and, in around 25% cases, epileptic seizures (Hagerman 1995, 1996). The gene implicated is referred to as *FMR1* and in unaffected individuals its 5′ untranslated region located in exon 1 contains about 30 copies of a CGG trinucleotide repeat. This repeat has intrinsic instability thought to result from a propensity to form an abnormal hairpin-like secondary structure which predisposes replication slippage; however, the somatic instability is relatively low compared to some other trinucleotide repeats such as the CAG sequence in the myotonic dystrophy gene (*DM1*). Instability can lead to increasing copy number of the repeat, which in the fully mutated state expands to contain more than 200 CGG repeats leading to the formation of a target

for extensive DNA methylation within the CGG repeat and in the flanking DNA (e.g. Sutcliffe et al. 1992; Hornstra et al. 1993). This in turn predicates the formation of altered chromatin structure, which can be manifested through specific treatments as a visible fragile site at Xq27.3 in metaphase chromosome preparations. The methylation and chromatin changes result in the cessation of *FMR1* expression and a lack of its protein product FMRP, which is responsible for the mental retardation phenotype in FRAXA. Fragile X mental retardation protein, FMRP, is a selective RNA-binding protein; it forms messenger ribonucleoprotein (mRNP) complexes which associate with polyribosomes in the brain and which can suppress protein translation in vitro (Laggerbauer et al. 2001) and in vivo (Li et al. 2001; Stefani et al. 2004).

Two intermediate stages are recognised before full expansion is reached; these are "intermediate" alleles (~41–55 repeats) and "premutations" (55–200 repeats). Both are now recognised as more than a generational stepping stone between normal allele size and the full mutation (Hagerman and Hagerman 2004). In addition, some studies have found an association between expanded alleles in these size ranges and cognitive/behavioural measures (e.g. Youings et al. 2000). Other studies indicate a significant negative correlation between allele lengths within the generally accepted normal range and a standardised score for IQ and general cognitive ability in young males, suggesting that modest increases in repeat numbers may have a limiting influence on cognitive performance (Loat et al. 2006). Interestingly, epigenetic modification and partial suppression of *FMR1* expression do not seem to be associated with phenotypes determined by such intermediate stages. Paradoxically, the intermediate alleles are associated with increased transcription, but lower levels of FMRP, and it seems that intermediate and premutation alleles manifest effects on the carrier by a distinct mechanism from the full mutation. Hagerman et al. (2001) have proposed an RNA "toxic-gain-of-function" model, whereby it is not an absence of protein, but rather an excess of RNA that causes the phenotypes associated with alleles in this range.

Recent studies have provided an insight into the possible mechanism underlying the hypermethylation of the expanded 5′ untranslated region of the *FMR1* locus. Naumann et al. (2009) have identified a methylation boundary existing at 650 and 800 nucleotides upstream of the CGG repeat, where the boundary separates a hypermethylated area from the normally unmethylated *FMR1* promoter, thereby protecting it from the spread of methylation. This boundary is lost in individuals with the fragile X syndrome and methylation extends into the *FMR1* promoter resulting in inactivation of the *FMR1* gene. It is speculated that the boundary in the *FMR1* 5′-upstream region coincides with a specific chromatin structure that, when destabilised, allows methylation to spread downstream, with concomitant repression of the *FMR1* transcript.

Other epigenetic changes accompany the hypermethylation of the expanded repeat. Use of immunoprecipitation approaches to analyse the histone patterns in the *FMR1* region near the expanded repeat revealed high levels of methylated histone 3 at Lys9 (H3K9me), lower levels of acetylated (Ac)H3 and H4 together with histone H3-K4 demethylation, suggesting that, unlike their normal-length counterparts, expanded CGG repeats at this locus carry epigenetic marks typical of

condensed heterochromatin (Oberlé et al. 1991; Coffee et al. 1999, 2002; O'Donnell and Warren 2002). Intermediate expansion may sometimes reflect the partial transition to heterochromatinisation, and some individuals with 60–200 CGG repeats display variable patterns of DNA methylation in different tissues (Saveliev et al. 2003). More recently, an intriguing novel gene, *ASFMR1*, has been identified. The *ASFMR1* transcript overlaps the CGG repeat region of the *FMR1* gene in the antisense orientation and is elevated in peripheral blood leucocytes of individuals with premutation alleles, but not expressed from full mutation alleles, suggesting that the locus may contribute to pathogenesis in premutation individuals (Ladd et al. 2007).

Primary and transformed cell cultures obtained from rare individuals of normal phenotype with an unmethylated full mutation in the *FMR1* gene have been used to show that CGG expansion per se does not block transcription (Smeets et al. 1995; Pietrobono et al. 2005).

There is a well-established parent-of-origin effect on the expansion of the CCG repeat in which asymptomatic mothers carrying permutation alleles produce male offspring with full expansions in all tissues (apart from testes in which contraction may occur). This results from repeat expansion in the maternal germline. Additional evidence indicates that changes in repeat copy number can also occur early in embryogenesis, which is particularly well illustrated by the observation of monozygotic (MZ) twins with different repeat lengths (Dion and Wilson 2009). The somatic instability of CGG repeats is low in patients with extensively methylated full mutations; however, unmethylated large expansions in FRAXA males who express *FMR1* show a high degree of instability (Wöhrle et al. 1998). Eiges et al. (2007) investigated the impact of differentiation on the methylation process in a model system based on embryonic stem cells derived from a male pre-implantation fragile X-affected embryo whose mother was a carrier of a premutation (170 repeats). The undifferentiated male cell line was shown to have expanded repeats in the range 200–> 1,000; however, the 5′ CGG expansion was unmethylated and the FMR1 protein expressed. Methylation and histone modification of the region, however, occurred once the stem cells were manipulated to differentiate. On differentiation, the promoter region, originally enriched in acetylated histone H3 and unmethylated at H3K9, on differentiation, showed loss of acetylated histone H3 together with increased methylation at H3K9. It seems that the critical stages of transcription-downregulation of the expanded repeat at this locus are coincident with the epigenetic changes occurring on cell differentiation.

In recent studies employing matrix-assisted laser desorption/ionisation time of flight mass spectroscopy (MALDI-TOF MS), Godler et al. (2010) have identified a number of novel elements at the FMR1 locus. The methylation of two of these termed FREE1 and FREE2 appeared to be highly correlated with the methylation of the CpG island and repression of gene activity in lymphocytes in blood from partially methylated "high functioning" full mutation males. Methylation of both markers in blood DNA from carrier females appeared to be inversely correlated with the *FMR1* activation ratio.

### 7.7.2 Monoamine Oxidases A and B

The neurotransmitter metabolising enzymes MAOA and MAOB play an important role in the metabolism of biogenic amines in the central nervous system and in the periphery. They are the products of closely similar and abutting genes arranged in a tail-to-tail configuration on the short arm at Xp11.23. Although both are capable of metabolising neurotransmitter amines, MAOB is also active towards dietary amines. As a consequence, in the context of behaviour, monoamine oxidase A (MAOA) has received detailed attention concerning its expression and inactivation status mainly because of its perceived significant role in aggression. This was first recognised in humans through observations on a family segregating an allele with a nonsense mutation that was associated with extremely disturbed and violent behaviour in affected males. Apart from this very rare deleterious mutation, there are two common alleles in most populations characterised by the presence of either three or four copies of a 30 base pair VNTR found within the promoter region of the gene and which are respectively associated with low *vs*. high transcriptional activity. This is of particular interest given the observations of gene by environment effects, which indicate that males carrying the low activity variant appear to be more vulnerable to childhood abuse and as a result are more likely to be involved in violent and antisocial behaviour, thereby perpetuating the "cycle of violence" (Caspi et al. 2002). Many subsequent studies have generally supported the interaction between adverse environments and the low activity allele, although different conclusions have been reached by some, depending on the sex, age and behavioural measures employed (Craig 2007; Craig and Halton 2009, 2010). The potential impact of epigenetic modifications to genes encoding members of the HPA axis and monoamine metabolism pathways (including monoamine oxidases), which may result from early exposure to stress are therefore of intense interest. Similarly, the inactivation statuses of the monoamine oxidases have been the object of several investigations. Some preliminary studies suggested that MAOA escaped (at least partly) from inactivation. This, however, was inconsistent with observations on allele differences in methylation of its CpG-rich regions and was also in conflict with studies examining RNA expression in myoblasts. By employing a PCR-based transcription assay on cloned myoblasts, which has the advantage of directly assessing transcription rather than the passive measure of methylation, Benjamin et al. (2000) showed that clonal populations expressed (at the level of detection on agarose gels) only either one or the other allele, which strongly suggests that the locus is regularly inactivated.

Pinsonneault et al. (2006) in a series of detailed investigations have examined the potential roles of inactivation and *cis*-acting regulatory factors on the expression of MAOA in human brain tissues. By employing two SNPs expressed at the mRNA level, they were able to evaluate relative allelic expression in females heterozygous for the high and low expressing VNTR genotypes (expressed as allelic expression imbalance ratios, AEI). Evidence of significant *cis*-acting effects was demonstrated by the wide range of AEI observed (0.3–4) in prefrontal cortex. However, although

extensive methylation in the promoter region VNTR was observed in females, but not males, this did not correlate with inactivation ratios as determined by an independent assay at the androgen receptor locus (Allen et al. 1992), which the authors took to indicate the existence of an alternative process of dosage compensation in females. Methylation at two CpG sites was examined in detail and showed extensive variation, but also correlated to some extent with allelic expression ratios. Local sequence also contributed to expression differences and it seems that allele-specific expression of MAOA in females depends on both genetic and epigenetic phenomena.

The impact of epigenetic programming coupled with the inactivation status of the MAOA locus may contribute significantly to the variable reports of association of low activity MAOA genotype and aggression in females. Very recently, a detailed bioinformatics study of the upstream region of the MAOA gene has provided valuable insight into the potential epigenetic modulation of expression from the locus and shown that the regulatory region extends up to 2,000 bp upstream of the transcription start site and contains a variety of potential methylation-sensitive regions (Shumay and Fowler 2010). In addition to the well-recognised VNTR, the analysis predicts a second putative promoter region whose activity is supported by the reported detection of a transcript (BC044787) apparently initiated within a CpG island distal to the conventional promoter. These data, taken together with the previous reports, suggest the potential for additional levels of regulation and epigenetic modulation of the control of MAOA expression in addition to those currently recognised.

There has also been some debate regarding the inactivation status of monoamine oxidase B (MAOB), whose levels in platelets provide a readily accessible source for monitoring its enzymic activity in various disorders. MAOB levels are lower in Turner's syndrome (X0) individuals than in control females (Good et al. 2003), providing suggestive evidence that the gene may escape inactivation and consequently that haploinsufficiency in X0 individuals may contribute to the range of symptoms observed in this condition. Subsequent investigations, however, failed to find an association between measured enzyme activity and cognitive skills in normal and X0? individuals (Lawrence et al. 2006). Chip-based expression studies as described above also do not provide any support that the locus escapes from inactivation.

### *7.7.3 Methyl-CpG-Binding Protein 2, MECP2 and the Chromatin-Modifying Gene, ATRX*

The role of *MECP2* in chromatin and genome modifications is of particular interest. This X-linked locus is itself subjected to epigenetic modification as well as having a central role in binding to methylated CpG signatures of other loci. Its significance in

context of behaviour is highlighted by its key role in Rett syndrome, RTT, which is one of the most common forms of intellectual disability in young girls. RTT-affected individuals manifest a progressive neurodevelopmental disorder which may include loss of speech, acquired microcephaly, ataxia and growth retardation. The disorder normally results from germline mutations in the gene and the almost exclusive involvement of females is best explained by X-linked dominant inheritance with lethality in the hemizygous males. Because of the normal involvement of MECP2 protein in binding to 7-methyl cytosine residues, it has been speculated that mutations may lead to a failure of binding leading to derepression of otherwise inactive loci; however, the spectrum of loci that it appears to influence is complex (Shahbazian and Zoghbi 2002).

Chahrour et al. (2008) examined gene expression patterns in the hypothalamus of mice that either lack or over-express *Mecp2*. In both conditions, *Mecp2* dysfunction induced changes in the expression levels of thousands of genes, but unexpectedly, the majority of genes (about 85%) appeared to be activated rather than repressed by Mecp2. The authors then selected a subset of six genes (*Sst*, *Oprk1*, *Mef2C Gamt*, *Gprin1* and *A2bp1*) and confirmed that Mecp2 binds to its promoters. In addition, they showed that Mecp2 associates with the transcriptional activator Creb1 at the promoter of an activated target, but not a repressed target. Their studies overall suggested that *Mecp2* regulates the expression of a wide range of genes in the hypothalamus and that it can function as both an activator and a repressor of transcription.

Chromatin immunoprecipitation studies indicated that the regulatory protein encoded by the early growth response 2 gene, *EGR2*, bound to the *MECP2* promoter and that MeCP2 bound to the intron 1 enhancer region of *EGR2* (Swanberg et al. 2009). These authors also noted that post-mortem cortex samples of both RTT and AS disorders showed decreased EGFR2 compared to matched controls and proposed a role for disruption of a pathway embracing these two regulators in both RTT syndrome and AS. Other studies on male mouse embryonic fibroblast cells deleted for *Mecp2* showed reduced levels of the cytoskeletal related gene, neuronal alpha tubulin, *Tuba1a*, and a deteriorated morphology. These defects were reversed by the introduction and expression of the human *MECP2* gene, suggesting that the latter is involved in the regulation of neuronal alpha tubulin, which had been shown to be reduced in brain tissue from both RTT and AS patients (Abuhatzira et al. 2009).

*MECP2* is expressed at high levels in post-mitotic neurons and one of its well-characterised and highly significant targets in the context of behaviour is the brain-derived neurotrophic factor gene, BDNF, which is important in regulating neuronal plasticity (Martinowich et al. 2003; Dulac 2010).

The MECP2 protein binds to methylated CpG dinucleotide sites through an 85 amino acid methyl-CpG-binding domain (amino acids 78–162) (Lewis et al. 1992; Nan et al. 1993) and mediates gene silencing by causing changes in chromatin structure through interactions with co-repressor complexes, such as SIN3A, which contains the histone deacetylases HDAC1 and HDAC2 – an interaction that is targeted through a 104 amino acid transcriptional repression

domain (amino acids 207–310) (Nan et al. 1993). MECP2 may also interact with other chromatin-modifying enzymes, such as histone methyltransferase and the product of another X-linked gene, *ATRX* (helicase 2, X-linked), mutations in which cause the alpha-thalassaemia/mental retardation syndrome (Nan et al. 2007; Shahbazian and Zoghbi 2002). Interestingly, MECP2 also interacts with the DNA methyltransferase, DNMT1, and consequently could be involved in the regulation of DNA methylation. In addition to its role in interacting with CpG islands, recent studies have shown that MECP2 can bind to intergenic sites and raise the possibility that its role may also include long-range chromatin reconfiguration (Yasui et al. 2007)

### *7.7.4 MECP2 and Its Association with ATRX Another X-Linked Chromatin Interacting Protein*

MECP2 has been implicated in the regulation of parentally imprinted genes – for example, by binding to the paternal allele of *H19* and the maternal alleles of *U2af1-rs1* (e.g. Drewell et al. 2002). Indeed, very recently, it has been observed that MECP2 and ATRX together with cohesin interact and bind to established imprinted domains such as the H19 imprinting control region and the Gtl2/Dlk1 imprinted domain in mouse brain. It is proposed therefore that ATRX, cohesin and MeCP2 cooperate to silence a subset of imprinted genes in the postnatal mouse brain (Kernohan et al. 2010). Furthermore, chromatin immunoprecipitation experiments have indicated that ATRX decorates the single late replicating (inactivated) X chromosome in female somatic cells (trophoblastic stem cells) but only after differentiation onset, as illustrated by its absence from embryonic stem cells. This suggests that the association of the chromatin remodelling protein follows the onset of inactivation and may be significant in the observed skewed X-inactivation observed in patients with ATRX syndrome (Baumann and de la Fuente 2009).

MECP2 mutations have also been observed in a wide variety of other behavioural disorders including autism, bipolar disorder and schizophrenia. Interestingly, milder late-onset versions of Rett syndrome have been attributed to skewed X-inactivation in which the mutant allele is significantly underexpressed (Amir et al. 2000). Mutations in coding and flanking regions of *MECP2* have been observed in autism at low frequency; more frequently, significant increase in promoter methylation associated with decreased expression (detected by immunofluorescence with anti-C-terminal MECP2 antibodies detecting both isoforms) has been observed in the frontal cortex of affected males by bisulphite sequencing. This suggests that the *MECP2* locus is affected by and itself influences the epigenetic programme with potential complex consequences (Nagarajan et al. 2006). The recent demonstration that there may be more common population variants affecting *MECP2*, which

contribute to the complex aetiology of autism may be relevant in this context (Loat et al. 2008a).

## 7.8 Skewing of Inactivation as a Novel Approach to Identifying Behaviours with X-Linked Genetic Input

As noted previously, a variety of mechanisms, both stochastic and directed, may lead to skewed X chromosome inactivation patterns in females, with modest to extreme skewing observed in a significant proportion of women. Extreme bias can additionally occur through cellular selection caused by deletions and translocations on one of the X chromosomes. It is interesting to note that differences in skewing between twin girls can also arise. Indeed, dichorionic MZ twinning, unlike monochorionic MZ twinning, occurs prior to the onset of X-inactivation (Puck 1998) and may lead to a relative increase in skewing of the former. Whatever the mechanism, skewing of X-inactivation should be expected to cause female MZ twins to be, on average, more discordant than their male homozygous twin counterparts for polymorphic X-linked traits. Given that the human X chromosome holds about 880 known protein-coding genes and that an impressive level of genetic heterogeneity exists, such that, on average, each gene may exhibit one or more single nucleotide polymorphisms (SNPs) and a scattering of variable simple sequence repeat motifs, the potential for functional heterozygosity at each locus and the potential for detecting X-linked traits by this process are considerable. In a preliminary study, Loat et al. (2004) examined nine behaviours or composite behavioural traits in 1,000 MZ female and 1,000 MZ male twin pairs taken from the Twins Early Development Study (TEDS) cohort. They found three of the traits (peer problems, prosocial behaviour and verbal ability) had significantly lower correlation coefficients for the female twins compared to males. They also found the same behaviours were significantly less correlated for dizygotic (DZ) male twins, which is to be expected as females share a common paternal X chromosome, so that any variation engendered by the differential inheritance of the two maternal X chromosomes will be moderated by the presence of the common paternal X. In contrast, males will be fully exposed to any functional variation for such traits resulting from the inheritance of one, or other, of the maternal X chromosome. Furthermore, the fact that they are hemizygous for X-linked genes which have no Y homologue means that the male sex as a whole may generally be exposed to extremes of phenotypes that are controlled by alleles on the X chromosome. This may help to explain the commonly recognised, yet infrequently discussed, phenomenon that males exhibit greater variance in the population than females for a large number of traits including intelligence (Hedges and Nowell 1995; Johnson et al. 2008; Craig et al. 2009). Subsequent studies have replicated these findings on older children and the additional data from these studies provided evidence that there are also X-linked QTLs for the social incapacity element of individuals with autism spectrum disorders (Loat et al. 2008b).

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# Chapter 8
# The Strategies of the Genes: Genomic Conflicts, Attachment Theory, and Development of the Social Brain

**Bernard J. Crespi**

**Abstract** I describe and evaluate the hypothesis that effects of parent–offspring conflict and genomic imprinting on human neurodevelopment and behavior are central to evolved systems of mother–child attachment. The psychological constructs of Bowlby's attachment theory provide phenomenological descriptions of how attachment orchestrates affective-cognitive development, and patterns of imprinted-gene expression and coexpression provide evidence of epigenetic and evolutionary underpinnings to human growth and neurodevelopment. Social-environmental perturbations to the development of normally secure attachment, and alterations to evolved systems of parent–offspring conflict and imprinted-gene effects, are expected to lead to specific forms of maladaptation, manifest in psychiatric conditions affecting social-brain development. In particular, underdevelopment of the social brain in autism may be mediated in part by mechanisms that lead to physically enhanced yet psychologically underdeveloped attachment to the mother, and affective-psychotic conditions, such as schizophrenia and depression, may be mediated in part by forms of insecure attachment and by increased relative effects of the maternal brain, both directly from mothers and via imprinted-gene effects in offspring. These hypotheses are concordant with findings from epidemiology, attachment theory, psychiatry, and genetic and epigenetic analyses of risk factors for autism and affective-psychotic conditions, they make novel predictions for explaining the causes of psychosis in Prader–Willi syndrome and idiopathic schizophrenia, and they suggest avenues for therapeutic interventions based on normalizing alterations to epigenetic networks and targeting public-health interventions toward reduction of perturbations to the development of secure attachment in early childhood and individuation during adolescence.

**Keywords** Attachment · Autism · Genomic imprinting · Schizophrenia · Social brain

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_8,
© Springer-Verlag Berlin Heidelberg 2011

## 8.1 Introduction

Conrad Waddington coined the term "epigenetics" to refer to the processes, whereby interactions between genetic and environmental variation lead to the emergence of patterns in phenotypic variation via development (Haig 2004a). In 1957, Waddington explicated in detail his theory of how natural selection and other processes mediate the evolution of ontogenetic trajectories, described by the concept of progressively increasing canalization as depicted in adaptive-developmental landscapes. His "strategy of the genes" thus centers on the evolution of cooperative developmental-genetic networks that can, in part via epigenetic modifications, produce relatively viable phenotypes in response to, and in spite of, genetic and environmental perturbations due to alterations in genes or their patterns of expression.

Epigenetic landscapes represent metaphors for how gene regulation directs pathways of development from the largest to smallest scales – from the first deviation in totipotency of embryonic stem cells, to gene-expression signatures, cell fate determinants and tissue-type differentiation into organs, and to phenotypes that include conditional behavior (Fig. 8.1). Much of modern developmental genetics focuses on elucidating the causal processes that underlie subsections of such landscapes, in the contexts of human ontogenies and disease, yet epigenetics itself, the impetus for Waddington's conception of development, has only recently

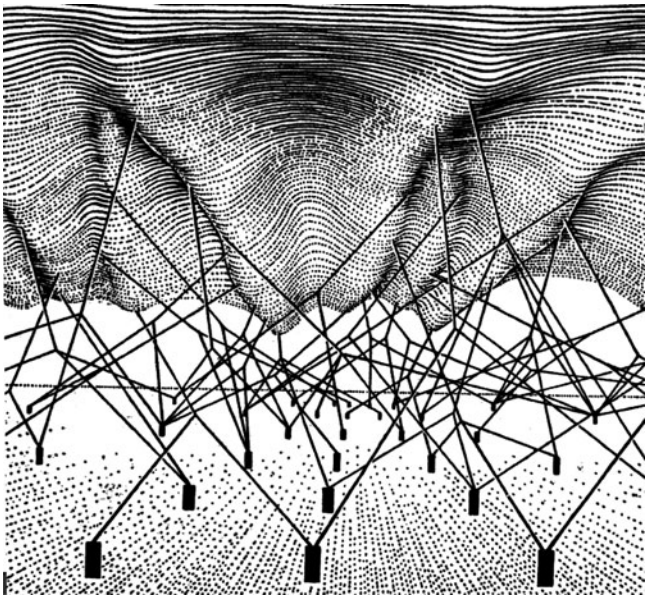

**Fig. 8.1** Waddington's (1957) conceptualization of an epigenetic landscape, with the pegs as genes and the strings as developmental effects from gene expression

reemerged as a central focus of research in understanding the orchestration of phenotypic development.

For humans, development proceeds via growth and differentiation of body, brain, and behavior under the joint influences of genes and, for environment, mainly interacting humans. Throughout much of the most formative early years, the mother holds center stage, providing both physiological nurture from uterus, placenta, and breast, and, increasingly, as the infant grows, psychological guidance through interactive processes of bonding, instruction, and other behavior. These developmental processes, perhaps more than many others, are expected to be strongly canalized, yet also sensitive to relatively predictable perturbations, such that epigenetic modifications to gene expression should direct neurodevelopment along conditionally adaptive pathways unless perturbations are too severe. As such, and as Waddington (1957) described, epigenetic landscapes may be considered as structures shaped by long evolutionary histories of selection, representing in their form some more or less long-term integration of past social environments and genomes.

From Waddington's mid-twentieth century perspective, the adaptive strategies of all genes in an individual coincide, and aspects of the environment, such as the agents of other genomes, are not expected to pursue strategies that conflict. Two levels and forms of genetic conflict are now well documented in their effects: conflicts between individuals who are genetically unrelated to some degree, and conflicts within the genomes of individuals, between sets of genes with different patterns of inheritance and genetic relatedness to potential interactants (Hamilton 1964, 1996; Trivers 1974; Haig 1997, 2006a, b; Burt and Trivers 2006; Fox et al. 2010). Both forms of conflict lead to differences in the phenotypic optima toward which they are, and have been, directed by natural selection. Optimal developmental trajectories of a given individual, and thus epigenetic landscapes, may thus vary in systematic ways between mother and child, and between sets of genes, such as imprinted genes that are silenced when inherited from either the mother or the father (Haig 2002). From evolutionary theory, and now-abundant empirical data (e.g., Hrdy 1999; Maestripieri 2002), offspring are expected to solicit more investment from parents, especially mothers as the main caregivers, than parents have been naturally selected to provide, due ultimately to parent–offspring relatedness of only one-half for autosomal genes. Similarly, paternally expressed imprinted genes in the child are expected to exert phenotypic effects that extract more investment from the mother than do maternally expressed imprinted genes, due to a history of paternally inherited genes exhibiting lower genetic relatedness within sibships and matrilines (Haig 2002, 2004b).

Outcomes of conflict between individuals, and between sets of genes, are difficult to predict, but may include stable equilibria, tugs-or-war over resource allocation, one party "winning" due to asymmetries in control over resource allocation, or continued conflict (e.g., Royle et al. 2004; Smiseth et al. 2008). Conflicts such as genomic imprinting also potentiate liability to phenotypes associated with disease (Crespi 2010), due to functional haploidy of imprinted genes, dysregulation of tug-of-war-based systems that evolved in this context, and the expected general higher lability of epigenetic gene-expression control systems (based on DNA methylation and histone

modifications) compared to the lability of DNA alterations via mutation. Such disease effects from imprinting have been documented extensively for disorders related to human placentation, overall growth, and neurodevelopment (e.g., Angiolini et al. 2006; McMinn et al. 2006; Wagschal and Feil 2006; Davies et al. 2008a, b).

The main thesis of this chapter is that genomic conflicts and cooperation, especially the epigenetic effects of genomic imprinting, centrally mediate core aspects of mother–child developmental interactions – most notably the processes of attachment – with important consequences for psychological well-being throughout life. I first describe the roles of imprinted genes in development, and recent discoveries of imprinted-gene networks that control growth. Next, I explicate the hypothesis that human early-childhood social development, mainly via interaction with the mother, involves a network of brain-expressed imprinted genes that modulate attachment – the process whereby children, in environments characterized by secure and responsive maternal care, internalize psychological constructs derived from external mother–child interactions to develop a self and psyche centered in their social context (Bowlby 1969; Bretherton 1997). The idea of imprinted genes mediating attachment was originally suggested by Isles and Holland (2005), and I extend and evaluate the hypothesis using information from patterns of imprinted-gene expression and coexpression, phenotypes of imprinting-based disorders, Bowlby's attachment theory, and psychiatric conditions involving the social brain. Finally, I discuss the implications of these ideas and data for pharmacological and behavioral therapies, public-health strategies, and the integration of epigenetic perspectives derived from Waddington into research programs focused on understanding the genetic bases of human development. Most generally, I integrate and synthesize evidence from evolutionary biology, developmental psychology, human genetics and epigenetics, and psychiatry of social-brain disorders to develop and evaluate explicit, testable hypothesis regarding roles of genomic conflicts and epigenetics in human development and evolution.

## 8.2 Genomic Imprinting in Human Growth

Haig and Graham (1991) developed the kinship theory of imprinting in the context of conflict interactions in fetal mice between paternally expressed *Igf2*, which drives growth, and maternally expressed *Igf2r*, which acts as a "decoy" receptor to reduce *Igf2*'s growth-stimulating effects. Although the predicted pattern of paternally expressed imprinted genes tending to foster overall growth, and maternally expressed imprinted genes constraining it, has been abundantly supported in studies of placentation and body size (e.g., Plagge et al., 2004; Weinstein et al., 2004; Kelsey 2007), the simple, direct tug-of-war system exemplified by *Igf2* and its receptor has proven to be atypical of imprinted-gene effects generally. The first clear evidence for a much more extensive system of imprinted-gene interaction – imprinted-gene networks – emerged from work by Arima et al. (2005), who demonstrated that the imprinted genes *ZAC1*, *LIT1*, and *CDKN1C* jointly mediate

growth of human cells. Varrault et al. (2006) used information from experimental mouse knockouts, and microarray databases, to document more directly the existence of a coregulatory imprinted-gene network; thus, gains and losses of *Zac1* altered the expression levels of the imprinted genes *Igf2*, *H19*, *Cdkn1c*, and *Dlk1*, and a broad pattern of imprinted-gene coexpression, involving these five genes as well as *Grb10*, *Gtl2*, *Peg1* (*Mest*), *Sgce*, *Dcn*, *Gatm*, *Gnas*, *Ndn*, and *Peg3*, emerged from analyses of gene coexpression in pooled datasets from mouse muscle and other tissues. Lui et al. (2008) subsequently identified a set of 11 imprinted genes in this network, all of which influence aspects of cell proliferation, whose expression levels across multiple tissues paralleled trajectories of overall body growth, and Gabory et al. (2009) showed that *H19* acts as an important transregulator of this imprinted-gene network that may also "fine-tune" gene coexpression patterns to moderate effects from perturbations; network interactions among unrelated genes are indeed postulated as a major cause of robustness against mutations (Wagner 2000), which should be especially important for functionally haploid, imprinted genes. Gabory et al. (2009) also demonstrated that such regulation via *H19* apparently did not operate in the placenta, which implies a notable degree of tissue and stage specificity of imprinted-gene network dynamics. Most recently, loss of expression of the *ATRX* gene in mice has been shown to cause altered postnatal expression of a suite of imprinted genes including *Igf2*, *H19, Dlk1, Zac1*, and *Peg1*, as well as the Rett-syndrome gene *MeCP2*, suggesting a role for *ATRX* in transregulation of the imprinted-gene network (Kernohan et al. 2010) and corroborating effects of *MeCP2* expression in the regulation of imprinted genes (Miyano et al. 2008).

Two independent lines of data provide further evidence for fundamental roles of imprinted-gene networks in development. First, two of the best-understood human genetic syndromes, Beckwith–Wiedemann syndrome and Silver–Russell syndrome, are each mediated by alterations to different imprinted genes in the network, which convergently generate similar phenotypes involving, respectively, overgrowth or undergrowth (Eggermann et al. 2008; Eggermann 2009). Similar convergent effects, whereby different epigenetic or genetic alterations produce highly similar phenotypes, have also been described for Prader–Willi syndrome (Crespi 2008a) and Angelman syndrome (Jedele 2007; Crespi 2008a). Convergence from diverse genetic or epigenetic perturbations, to similar phenotypes across multiple traits, represents clear examples of developmental canalization. Such canalization effects can also be generalized to idiopathic conditions, such as autism and schizophrenia, each of which also exhibits diverse genetic, epigenetic, and environmental causes converging to a relatively small set of cognitive, affective, and behavioral phenotypes (Happé 1994; Owen et al. 2007; Abrahams and Geschwind 2008). As imprinted-gene networks have presumably evolved in part due to selection for coordinated, robust control of mammalian growth – yielding specific, syndromic phenotypes when sufficiently perturbed – genomic and epigenetic networks orchestrating human neurodevelopment and behavior may likewise be expected to yield predictable patterns from different forms of alteration.

A second line of evidence pertinent to gene-expression networks in general, and imprinted genes in particular, is the recent discovery of mechanisms, whereby

imprinted domains interact across different chromosomes, via allele-specific physical juxtaposition of long-range chromatin loops in interphase nuclei (Smits and Kelsey 2006; Zhao et al. 2006). Such interactions occur genome-wide (Ling and Hoffman 2007), but are strongly enriched to imprinted regions (Zhao et al. 2006), with an apparent central role for the imprinted RNA gene *H19* as a hub for transvection of parent-of-origin specific effects to both imprinted and non-imprinted loci on other chromosomes (Sandhu et al. 2009). Interchromosomal interactions that involve imprinted loci provide a genome-scale mechanism for coordinated expression of imprinted genes (in addition to mechanisms involving, for example, transcription factors such as *Zac1*, and protein–protein interactions such as those between *p57kip2* and *Nurr1*) (Joseph et al. 2003), and for control of non-imprinted genes and loci by specific imprinted genes, that may serve to increase their relative influence on development.

In the context of Haig's kinship theory of imprinting, imprinted-gene networks can be hypothesized as multidimensional generalizations of simple, two-dimensional tugs-of-war, which apparently evolved step by step with the accrual of imprinted domains along the lineages leading to the origins of metatherian and eutherian mammals (Renfree et al. 2009). This conception of imprinted-gene networks shows how cooperation, in a literal sense of the word, can evolve from conflict, when the interests of different mutually dependent parties, here paternally expressed and maternally expressed imprinted genes, overlap partially yet broadly. Thus, paternal and maternal genes, as well as additional genetic "factions" such as genes on the X chromosome (Haig 2006a), share a common interest in successful development via reducing physiological costs of conflict, and (within limits) increasing the robustness of development to perturbations, although natural selection continues to favor paternal-gene variants that solicit marginally more investment from mothers, and maternal and X-linked alleles that reduce such imposition of increased costs.

Consideration of the tissue and stage specifics of imprinted-gene expression, in the context of the kinship theory of imprinting, leads to the inference that more or less different imprinted-gene networks should characterize each of the three main arenas for imprinted-gene effects (1) placentation, (2) overall postnatal growth, and (3) behavioral interactions with the mother, as influenced by imprinted-gene expression in the brain. Indeed, the network presented in Varrault et al. (2006) represents a conglomeration of gene coexpression patterns across multiple tissues and stages, and some central genes in the network, such as *Ndn*, apparently do not exert effects on overall growth (Tsai et al. 1999). Eutherian placentation is known to be orchestrated by imprinted genes in a manner consonant with predictions of the kinship theory (e.g., Bressan et al. 2009), and alterations to imprinted-gene expression underlie a considerable proportion of risk for the major disorders of human pregnancy (e.g., Charalambous et al. 2007); Fauque et al. (2010) describe evidence for imprinted-gene coregulation effects in mouse placentation, which coordinate gene-expression changes in relation to early-embryonic conditions. Postnatal growth effects are similarly mediated by imprinted genes, which appear to predominantly exert their influences through effects on cell proliferation at the tissue level (Reik et al. 2001), and glucose and lipid metabolism at the levels of physiology and metabolism (Sigurdsson

et al. 2009). The body growth enhancement effects of Beckwith–Wiedemann syndrome, and growth reduction in Silver–Russell syndrome, indeed represent paradigmatic examples of imprinted-gene disorders with primary effects on both prenatal and postnatal growth. Microarray studies that focus on placental gene expression, and gene expression across the tissues most directly involved in prenatal and postnatal growth, are thus expected to reveal imprinted-gene networks that comprise partially overlapping sets of genes, with imprinted genes exerting effects that are more or less tissue specific. The tissue with the most pervasive effects on growth, development, and behavior – the brain – remains, however, the least well understood.

## 8.3 Genomic Imprinting in the Brain

The study of imprinted-gene effects in brain development was pioneered by Keverne, whose studies of chimeric mice showed differential contribution of paternally expressed imprinted genes to development of limbic brain regions (especially the hypothalamus), and maternally expressed genes to development of the neocortex (Allen et al. 1995; Keverne et al. 1996; Keverne 2001a, b). Functional and evolutionary hypotheses for the effects of brain-expressed imprinted genes have been described in detail; these include diverse effects on affect, cognition, attention, feeding behavior, and other central brain functions (Isles et al. 2006; Wilkinson et al. 2007; Davies et al. 2008a, b; Champagne et al. 2009), some of which apparently influence resource-related interactions between mothers and offspring. These hypotheses have proved difficult to evaluate critically due to the complexity of the mechanisms involved, and the difficulty of testing alternative hypotheses of neurobehavioral adaptation compared to pleiotropic by-product.

Adaptation is most commonly recognized, at least initially, as convergence or parallelism in causal patterns consistent with theory. For imprinted brain-expressed gene, clear convergent effects of paternally expressed genes on neurodevelopment have been described for the roles of *Peg3, Peg1*, and *Ndn* in promoting development of hypothalamic neurons that secrete oxytocin, the peptide hormone that most strongly mediates social bonding (Davies et al. 2008a, b; Ross and Young 2009; MacDonald and MacDonald 2010). Moreover, in the *Zac1* network (Varrault et al. 2006), these three genes occupy central locations as "hubs", prominently connected, like *Zac1* itself, to relatively large numbers of both imprinted and non-imprinted genes. These findings, from three lines of evidence (mouse knockouts, functional gene-expression data, and gene coexpression networks), suggest a functional and evolutionary role for imprinted genes in fostering bonding and attachment of offspring to mothers, in the context of an imprinted-gene network that affects expression of offspring behaviors that regulate levels of resource accrual from mothers. In mice, bonding of pups to mothers involves olfactory, tactile, and auditory cues, which foster safety and suckling; moreover, suckling behavior is differentially disrupted in mouse knockouts of the paternally expressed genes *Peg3* and *Gnasxl* (Curley et al 2004; Plagge et al. 2004).

In humans, who like mice are highly altricial as neonates, attachment involves the same three categories of sensory cue as in mice, and early suckling and feeding are impaired in the two genomic conditions, Silver–Russell syndrome and Prader–Willi syndrome, that involve strong maternal-gene biases (Blissett et al. 2001; Holland et al., 2003; Dudley and Muscatelli 2007), as well as in humans with paternal deletion of *GNASxl* (Geneviève et al. 2005); in contrast, macroglossia (enlarged tongues, which are expected to facilitate suckling) has been reported in both Beckwith–Wiedemann syndrome and Angelman syndrome (Cohen 2005). But much more extensively than in mice, human cognitive-affective interactions guide early development of the child's "social brain" – the distributed, integrated set of neural systems that control acquisition, processing, and deployment of socially interactive information (Frith 2008). Such interactions have motivated the development of a large body of theory and empirical work in psychology and attachment theory, with direct implications for other fields from genetics, epigenetics, and neurodevelopment to analyses of the etiology of psychiatric conditions that involve alterations to early development and function of the social brain.

## 8.4 Attachment Theory and Human Social Development

Attachment theory was developed by John Bowlby and Mary Ainsworth to help explain the adaptive significance of physical and psychological interactions between young children and close caregivers (primarily the mother), and how children deprived of care, or subject to dysfunctional forms of caregiver–child interaction, develop along lifelong trajectories characterized by altered emotional and cognitive systems that are explicable in part by the nature of these early perturbations (Bowlby 1969). The majority of children develop "secure" attachment, whereby their intimate interactions with the mother provide for physical safety, nutritional sustenance, and social-emotion-cognitive guidance that generates a "secure base", increasingly explorative behavior with age, and an environmental conducive to development of a social brain – internalized schema – with robust sense of self, well-developed theory of mind, and ability to nurture secure attachment to one's own children in later life.

Deviations from secure attachment, as assayed by the "strange situations test" that tests a child's behavior toward its mother when subject to short separations, take a small set of forms (1) avoidant attachment, whereby children with unmet expectations of attachment security come to at least provisionally reject significant others, (2) anxious/ambivalent attachment, with unmet solicitation leading to "escalation" of distress and behavior characterized by contact-seeking combined with anger and ambivalence, and (3) disorganized attachment, with lack of a coherent, organized "strategy" for interacting with the caregiver (e.g., Shaver and Mikulincer 2002). These deviations have been interpreted in the context of developmental responses by the child to variation in caregiver sensitivity, that is, provision of consistent physical and psychological support, in contrast to neglectful, hostile, or inconsistent care.

The framework for interpretation of childhood attachment patterns has developed in the combined contexts of post-Freudian psychodynamics, evolutionary-ethological constructs for the study of animal behavior, and psychological theories of stages and processes in child social development, such as the social-behavioral internalization theories of Vygotsky (Bretherton 1992; Thompson 2008). In her book *Mother Nature*, Hrdy (1999) first conceptualized an evolutionary-genetic basis for understanding central aspects of attachment, unappreciated by previous work, that follow directly from W.D. Hamilton's inclusive fitness theory. Due ultimately to genetic relatedness between mothers and children of only one-half, mother–child interactions are expected to comprise complex mixtures of cooperation and conflict, with children having been selected to solicit higher levels of behavioral as well as nutritional investment than mothers have been selected to provide. The theory underlying parent–offspring conflict has been well supported by empirical studies (Bowlby 1969, p. 203; Haig 1993; Hinde and Kilner 2007), and Hrdy (1999) interprets a broad swath of human-specific childhood phenotypes, from high levels of neonatal fat, to precocious neurological development of eye contact, facial expression, and vocalization, as subject to a long history of selection in the context of child signaling of vigor and solicitation of increased energetic and psychological investment. Childhood attachment to mother – from placenta, to breast, and to psychological development – is thus expected to be centrally mediated by both cooperation and conflict, which should be expressed in patterns of child attachment behaviors that represent largely adaptive responses to the behavior of the mother, who is in physical control of investment. The major patterns of child attachment have indeed been interpreted by attachment theorists as conditionally adaptive responses of children to sensitive, hostile, neglectful, or inconsistent mothering, though not in terms of strategies grounded by the fundamentals of evolutionary genetic and epigenetics.

Parent–offspring conflict theory applies to autosomal genes with conditional, temporally restricted effects in children, and autosomal genes expressed in mothers. The primary implication of this theory is that evolved systems of mother–child interaction (and father–child interaction, given some degree of paternal care) should be characterized in part by conflicts – mainly centered on increased demands from the child, and responses from mother conditioned on marginal benefits to her from incremental investment in child versus benefits from other fitness-accruing activities. Haig (1993) describes evidence of such dynamics in the context of human maternal–fetal interactions, as do Wells (2003) and Soltis (2004), for suckling and crying; these authors also demonstrate how the major disorders of food provision via placenta and breast, including intrauterine growth restriction, pre-eclampsia, gestational diabetes, excess post-natal weight gain, failure to thrive, and colic, can usefully be understood in part as forms and effects of dysregulation to systems of evolved mother–child conflict – conflicts that are revealed most clearly in cases of genetic or environmental perturbation from "normal" development. Mother's primary form of investment in children beyond food is, of course, psychological training and guidance, and mother–child conflicts in this arena are expected to exhibit constrained-conflict dynamics squarely manifest in attachment,

with disorders of mental health, for the grown child, more likely to result when dynamics are perturbed (Berry et al. 2007, 2008; Lyons-Ruth 2008).

To understand the expected role of imprinted genes in attachment, conflicts due to imprinted genes must be conceptually distinguished from, and related to, conflicts between parents and offspring. Imprinted-gene conflicts are thus expected, from the kinship theory of imprinting, to involve interactions between paternally expressed genes and maternally expressed genes in the child. As such, imprinted genes are predicted, through the integration of their effects, to influence the child's "set level" of resource demand imposed upon the mother. An example of such an economic system, in a physiological situation, is provided by expression of the imprinted gene *Cdnk1c* in fetal mouse development: levels of its protein product, *p57kip2*, have been experimentally demonstrated to act as a "rheostat" for embryonic growth, with ultimate levels of growth determined by the balance between fetal demand and maternal supply (Andrews et al. 2007). In the context of child psychology and behavior, imprinted genes are expected to similarly mediate levels of imposed demand, here for psychological time and energy; more generally, they should exert effects that result, by any mechanism, in higher or lower, longer or shorter, levels of investment from the mother. Small alterations to such systems, toward, for example, stronger effects from paternal gene expression that lead to higher investment, are expected to benefit the child, and his or her paternally expressed imprinted genes, at a small cost to the mother and to the child's maternally expressed imprinted genes. In contrast, large alterations, which like any large genetic or epigenetic effects surmount the homeostatic effects of canalized development, are expected to result in disorders of psychological development that are detrimental to both mother and child, and to all genomic parties concerned. The nature of such disorders, however, provides useful tests of evolutionary theory involving genomically based conflicts, and insights into strategies for therapy, prevention, and research (Crespi 2008b; Crespi et al. 2009).

These considerations from attachment theory, parent–offspring conflict theory, and the kinship theory of imprinting generate a simple conceptual framework for understanding normal and dysregulated social-behavioral interactions between mothers and small children (Fig. 8.2). From birth, young children exhibit some level of social-behavioral "demand", manifest in solicitations of interaction via facial and auditory cues, as well as in the contexts of crying, fussing, and suckling. Such levels are expected to vary between children, and over time in response to levels and forms of reciprocal and unsolicited maternal behavior. Child-specific, dynamic "demand" is matched, less or more, by levels of maternal sensitivity and responsiveness to such cues – varying from neglect, to solicitous, attentive care, and to controlling over-involvement.

Evolutionary histories of parent–offspring conflict, and imprinted-gene conflict, have generated conditional behavioral adaptations, in child and in mother, that potentiate the possibility of mismatches between demand and response, which may result in maladaptation for one or both parties. Thus, relatively low social-behavioral demand from the child may delay or dysregulate social-brain development, but provide marginal benefits to the mother in terms of her lifetime inclusive fitness.

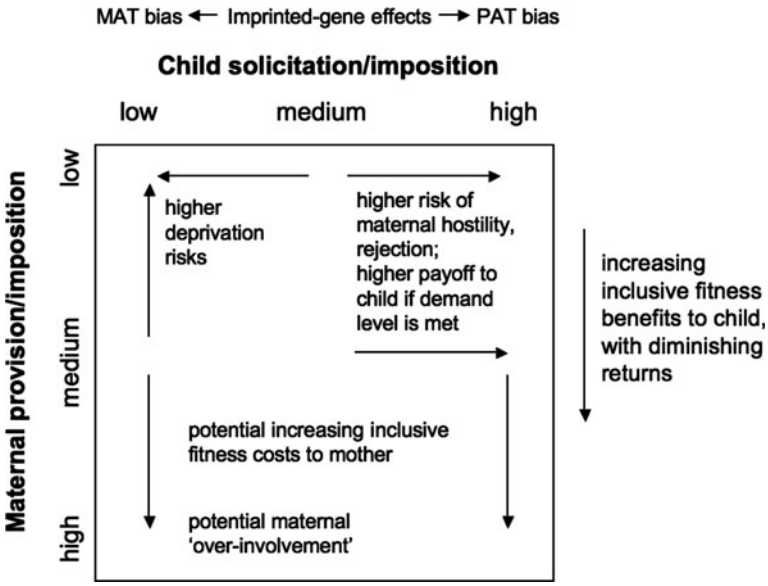


**Fig. 8.2** A simple model for potential interactions of levels of maternal social-behavioral provision/imposition with levels of child social-behavioral solicitation/imposition

In contrast, relatively high demands may, if matched by supply, provide benefits to the child at some cost to mother; if not matched or if too insistent, however, such demands may provoke neglect, ambivalence, or even hostility, if they interfere sufficiently with a given mother's perceived optimal life-history trajectory. Levels of maternal "supply" higher than child demand – "over-involvement" – may, in contrast, come to interfere with the child's ability to explore and increasingly detach, physically and social-emotionally, from its secure base of the controlling maternal brain, to individuate its own psyche. This conceptual framework can help to explain the surprisingly low rates of secure attachment in children (not much over 50%), with anxious attachment representing, in part, attempts to solicit increased investment and improve one's lot, whereas avoidant attachment may involve a form of defensive strategy to, in part, avoid making matters any worse.

The outcomes of parent–offspring and imprinting-mediated conflicts are difficult to predict a priori, but they should depend upon the relative fitness costs and benefits to the child and mother of the alternative behaviors, and the degree to which each party controls aspects of the interaction. Thus, tactics available to young children include auditory and facial expressions of positive and negative affect, crying, suckling intensity, and physical movement varying with age; in contrast, mothers control access to breast and physical presence, and provision of information and social interaction crucial to the child's affective and cognitive development. Indeed, as Bowlby (1951, p. 53) noted:

"*In dealing here with the embryology of the human mind one is struck by a similarity with the embryological development of the human body, during the course of which*

> *undifferentiated tissues respond to the influence of chemical organizers. If growth is to proceed smoothly, the tissues must be exposed to the influence of the appropriate organizer at certain critical periods. In the same way, if mental development is to proceed smoothly, it would appear necessary for the undifferentiated psyche to be exposed during certain critical periods to the influence of the psychic organizer – the mother*". Thus, during infancy and early childhood, "*she is his ego and his super-ego*."

The primary usefulness of a Keynesian, supply–demand model for attachment is that it provides a framework, compatible with both developmental psychology and evolutionary-genetic theory, that generates novel explanations and predictions for alternative patterns of social-brain development. The model can be conceptualized as a developmental landscape, canalized to secure attachment and its sequelae but with different optimal trajectories for mother and child, and for child's paternal and maternal imprinted genes. Child development along the landscape initially proceeds via basic emotional-behavioral attachment mediated in part by paternal imprinted-gene effects, which serve as an affective-attentional scaffold upon which the social, maternal brain is gradually built, via maternal-gene effects and interactions with the mother and other caregivers. Actual trajectories for any child are determined by some dynamic integration of genetic, epigenetic, and environmental inputs into social-brain development, and into mother–child interaction; most importantly for mental health, relatively large genetic, epigenetic, or environmental alterations to this system are expected to generate predictable consequences, manifest in major disorders of the social brain, such as schizophrenia, depression, and autism. The assumptions and predictions of the model can be evaluated using information from human imprinted-gene syndromes, and studies on the etiology of schizophrenia and autism, in the contexts of attachment and evolutionary-genetic theory.

## 8.5 Prader–Willi Syndrome

Haig and Wharton (2003) describe how the complacent nature of Prader–Willi infants (with low levels of crying and wakefulness), and their compulsive self-foraging for food after the usual age of weaning, can be interpreted in the context of reduced demands on mothers, as expected under imprinted-gene biases involving loss of paternal-gene expression. Prader–Willi syndrome is known to centrally involve hypothalamic alterations and relatively low levels of oxytocin (Swaab et al. 1995; Holland et al. 2003; Höybye 2004; Goldstone 2006), which is consistent with the oxytocin-reducing effects of *Ndn* gene knockouts in mice (Davies et al. 2008a, b) and the expected behavioral effects of lower oxytocin levels – reduced motivation toward social bonding behavior. These considerations suggest that Prader–Willi syndrome should involve insecure attachment in children, especially among cases due to maternal uniparental disomy in which the genomic deviations toward maternal imprinted-gene effects are more extensive. This suite of behavioral and other alterations in Prader–Willi syndrome may involve, in part, disruptions to genes that underlie a human-lineage trajectory toward earlier weaning and shorter

interbirth intervals (Sellen 2007; Haig 2009; Humphrey 2009), a transition expected to impose strong selection in the context of maternal-offspring and imprinted-gene conflicts given generally high rates of early-childhood mortality.

The extensions of attachment theory described here provide a simple, novel hypothesis to help explain one of the other major features of Prader–Willi syndrome: the extremely high levels of affective psychosis upon reaching adolescence (Soni et al. 2008; Webb et al. 2008). By this hypothesis, Prader–Willi infants and children may essentially undergo a process of self-induced maternal deprivation, whereby their low levels of care solicitation lead to reduced interaction with the mother and dysregulated social-emotional development. The primary line of evidence supporting this hypothesis, in addition to the theory and data described above, is that maternal deprivation, due to diverse causes including reduced physical care, temporary early separation, or permanent loss, is a well-documented, highly penetrant causal factor in the etiologies of depression (e.g., Pesonen et al. 2007; Tyrka et al. 2008; Liu et al. 2009), schizophrenia (reviewed in Read and Gumley 2008), and schizotypy (Anglin et al. 2008). Convergent evidence for interactions of maternal deprivation with imprinted-gene expression comes from a study of mice, where 2 (5.4%) of 37 genes significantly upregulated in the hypothalamic paraventricular nucleus of young mice in response to maternal deprivation were imprinted (*Gtl2* and *Ipw*), a higher proportion than expected given an approximate 1.2% (120 of 10,000) (http://igc.otago.ac.nz/ table.html) of brain-expressed genes imprinted in the mouse genome ($P < 0.05$).

To the extent that affective psychosis in Prader–Willi syndrome is mediated by gene–environment interaction in this context, alterations to early maternal-social environments, and facilitation of early bonding via behavioral and hormonal therapies, may help to alleviate the psychiatric sequelae of this large-scale epigenetic disruption. Similar considerations should apply to genetic and epigenetic alterations that yield phenotypes overlapping with Prader–Willi syndrome (Crespi 2008a) and, perhaps, to effects from reduced size of the paraventricular nucleus in patients with bipolar disorder and major depression, compared to controls (Manaye et al. 2005). More generally, an evolutionary trajectory toward earlier weaning and shorter interbirth intervals in humans might be expected to have increased vulnerability to affective-psychotic conditions, unless attachment through alloparents and fathers (Hrdy 2009) compensates for abbreviated maternal care.

## 8.6 Angelman Syndrome

In contrast to children with Prader–Willi syndrome, children with Angelman syndrome are highly active, with reduced duration of sleep, frequent suckling attempts, and enhanced levels of socially directed positive affect (Cohen et al. 2005; Oliver et al. 2007). These diverse phenotypes have been interpreted as reflecting increased behavioral and energetic demands on the mother, as predicted by the direction of imprinted-gene deviation, toward reduced relative effects from the maternally expressed imprinted gene *UBE3A* (Oliver et al. 2007). Such an

increased "set level" of demands imposed on mothers might be expected to lead to insecure forms of attachment, unless the demands are met, whereupon social-emotional development should proceed as normally as possible under the circumstances of severe intellectual disability that characterize this syndrome. A high prevalence of autistic traits in Angelman syndrome (Sahoo et al. 2006; Bonati et al. 2007; Jedele 2007) has also been interpreted in the context of selectively reduced social-brain development and high demands imposed on the mother in the context of paternal imprinted-gene bias (Brown and Consedine 2004; Isles et al. 2006; Crespi and Badcock 2008).

## 8.7 Schizophrenia and Attachment

Schizophrenia represents a set of conditions, due to diverse causes, with some combination of symptoms that may include hallucinations, delusions, thought disorder, flat affect, and anhedonia (Read et al. 2004a, b). An attachment-theory perspective on schizophrenia was first provided by Bowlby (1973, pages 174–177, 318–319), in his description of continuities between forms of abusive and dysfunctional psychological treatment of children by parents and the later development of specific schizophrenic symptoms. Systematic analyses of attachment in relation to aspects of schizotypy and schizophrenia (Berry et al. 2007, 2008; Gumley et al. 2008; Tiliopoulos and Goodall 2009) have documented higher than normal levels of insecure attachment in these conditions, with general support for Bowlby's prediction of linkages between childhood experiences and psychotic-behavioral profiles. Thus, in nonclinical populations avoidant attachment has been associated with negative schizoptypal traits, and anxious attachment with positive schizotypy (Wilson and Costanzo 1996; Berry et al. 2006); insecure attachment has also been linked with paranoia in schizophrenia (Pickering et al. 2008), and disorganized attachment may be related to dissociative cognitive states that accompany schizophrenia in some patients (Berry et al. 2008; Liotti and Gumley 2008).

One of the best-replicated patterns reported in families with an adolescent or young-adult schizophrenic offspring is high levels of "expressed emotion" – some mix of parental hostility, criticism, and over-involvement (Read et al. 2004a, b), which commonly occurs in the context of emotional over-involvement by one or both parents but low levels of actual care. Higher expressed-emotion predicts higher rates of relapse after a first psychotic episode and also appears to be linked with the initial development of schizophrenic symptoms themselves (Read and Gumley 2008; Read et al. 2004a, b). The causal bases for such associations have puzzled psychiatrists for many years, but perspectives from attachment theory and genomic conflict offer novel, testable insights. Thus, parental over-involvement might be expected to involve attempted control over offspring behavior, interfering with the usual processes of physical and emotional separation of the child from attachment figures, which becomes most pronounced in adolescence (Harrop and Trower 2001). Moreover, extended controlling influences on the offspring psyche

by the mother – the "maternal brain" of genomic imprinting theory (Badcock 2009, pages 154–157) – as expressed in the mother's behavior and in maternal-gene influences on development within her child – are expected to engender notably higher risk of schizophrenia. Such a prediction is broadly compatible with maternal imprinted-gene biases, and their correlates, being differentially associated with higher schizophrenia risk (Crespi 2008a; Crespi and Badcock 2008), but additional, targeted tests are required for robust evaluation. More generally, evolutionary theory provides clear, specific predictions regarding the presence and causes of conflicts within families (Emlen 1995, 1997; Surbey 1998), which underpin and complement psychodynamic and socioeconomic perspectives on such conflicts, as well as providing a basis for understanding conflicts within the minds of normal "individuals", and those beset with such conditions as schizophrenia or depression (Read et al. 2004a, b; Haig 2006b; Badcock 2009). As Laing and Esterson (1970, p. 12) inquired, "are the experiences and behaviour that psychiatrists take as symptoms and signs of schizophrenia more socially intelligible than has come to be supposed?"

Long-term prospective studies, using high-risk populations, are required to evaluate patterns of cause and effect in relating childhood experiences and attachment patterns to the risks and forms of depression, schizophrenia, and other conditions. Evolutionary theory provides a conceptual framework to guide such studies, a framework that is fully compatible with both a psychoanalytic focus on childhood social-brain development in the family context and a strong role for interactive genetic and epigenetic effects in modulation of risk. In this context, the mother represents the primary environment of the child from conception until well past weaning, an environment that intimately modifies gene expression, and neurodevelopment, in the growing child. As such, supportive public-health interventions during early child development and in adolescence, which are sensitive to the main causes of conflicts within families, are expected to yield disproportionate returns in reducing the incidence, severity, and recurrence of schizophrenia and depression, and more generally increase emotional well-being in society (e.g., Gumley et al. 2008).

## 8.8 Autism and Attachment

Idiopathic autism represents a spectrum of related conditions, with many genetic, epigenetic and environmental causes, characterized by differential underdevelopment of the social brain from earliest infancy (Kanner 1943; Happé 1994; Abrahams and Geschwind 2008). A central focus in attachment-theory research on autism has been whether or not autistic children can indeed develop secure attachment with the mother, given that Kanner's (1943) original description of autism posited lack or disturbance of affective contact as a central feature. Rutgers et al. (2004) reviewed 16 studies of attachment in autistic children, finding that autism was associated with lower levels of secure attachment overall, but that this

difference "disappeared in samples of children with higher mental development" or less severe symptoms, suggesting that attachment security is compatible with at least relatively high-functioning autism. In contrast, van Ijzendoorn et al. (2007) and Rutter et al. (2009) question the applicability and validity of attachment theory itself to autism, given the unusual nature of social relationships in this condition and uncertainties concerning whether autistics can develop internal working models of self and others, which form the bases for linguistic discourse. Psychological perspectives based in Vygotsky's work indicate, for example, that linguistic interactions with mother mediate childhood progress from listening, to private speech, and eventually to inner speech as thoughts (Fernyhough 1996), a process that is absent or underdeveloped in autism but may by contrast, be dysregulated via "re-expansion" in the auditory hallucinations of schizophrenia (Fernyhough 2004).

A general view of autism posits that under-development of the social brain should, as described by Kanner, centrally involve weak social-emotional connections with other individuals. Such differential social deficits, however, need not preclude relatively basic forms of attachment to the mother, as described by Bowlby for mother–offspring relations across most mammals. Indeed, from the perspective of parent–offspring conflicts and genomic imprinting, some subset of autism cases might also be considered to involve pathological overexpression of increased levels of demand imposed on mothers by offspring (Badcock and Crespi 2006; Crespi and Badcock 2008). Such demand may be imposed either directly, through behaviors that solicit forms of investment as in Angelman syndrome, or indirectly, through self-oriented, nonsocial behavior that precludes or delays physical independence, requiring mothers or others to provide longer-term, more highly intensive care. This hypothesis is concordant with several lines of evidence, including (1) accelerated brain and body growth in young children with autism and increased relative effects from paternally expressed imprinted genes (Crespi and Badcock 2008), (2) imprinted-gene effects on reaction to novelty, and dispersal, in mice (Isles et al. 2002; Plagge et al. 2005), (3) a higher incidence of autism in males, who are more costly than females to rear (Gibson and Mace 2003; Rickard et al. 2007; Tamimi et al. 2003), (4) myriad reports of close and sustained, if atypical, relationships between mothers and their autistic children (e.g., Hoffman et al. 2009), and (5) temperaments of autistic children that involve higher rates of activity, impulsivity, and noncompliance (e.g., Garon et al. 2009).

The degree to which autism spectrum traits in nonclinical populations actually engender higher levels of early maternal or biparental investment has yet to be investigated, and indeed, many childhood social abilities are expected to have evolved in the context of soliciting higher investment from mother via providing her with positive-affect emotional benefits contingent on social skill development (e.g., Hrdy 1999), in addition to imposing negative-affect costs through behaviors such as refusal, tantrums, and, perhaps, insistence on sameness. For analyzing behavioral interactions between mothers and offspring, it is crucial to bear in mind that "disorders" such as autism are postulated to represent sequelae to dysregulation of mechanisms that evolved in contexts of both conflict and cooperation, with evolutionary and behavioral conflicts potentiating increased levels of risk (Crespi 2010).

A primary implication of attachment theory, and behavioral-evolutionary genetics, for elucidating the causes of autism, is that early-childhood social motivation, in the context of "demands" imposed on mothers via physical, social-emotional, and linguistic interactions, should drive cascading effects on social-brain development that are either normal or deviate toward autistic phenotypes. The neurological and endocrine bases for social motivation and affective bonding in infants (e.g., Bowlby 1969 p. 203–204; Moriceau and Sullivan 2005; Grossmann et al. 2008) and children (e.g., Bartz and Hollander 2008) have been much less studied than those in mothers (e.g., Strathearn et al. 2009), though their relevance for understanding mother–child interactions should be no less consequential. Studies of plasma oxytocin levels in autism have yielded unusual results: two studies found lower plasma oxytocin in children with autism (Modahl et al. 1998; Green et al. 2001), but unexpectedly, oxytocin levels were positively associated with degree of social impairment (Modahl et al. 1998); in the single study of adults, autistic individuals showed significantly higher levels of plasma oxytocin than did controls (Jansen et al. 2006). Might oxytocin in autistics subserve, to some degree, positive reinforcement of nonsocial stimuli, or might epigenetically based reduction of oxytocin receptor expression (Gregory et al. 2009) follow from early social-interaction deficits?

The μ-opioid system represents another important candidate mechanism, in addition to oxytocin, for neuroendocrine regulation of attachment, given links of allelic variants in the μ-opioid receptor gene *OPRM1* with attachment patterns, sensitivity to social rejection, drug dependence, and risk of schizophrenia (Insel 2003; Barr et al. 2008; Way et al. 2009; Mague and Blendy 2010; Serý et al. 2010) and evidence for parent-of-origin effects influencing phenotypic effects of this gene (Lemire 2005).

## 8.9 An Imprinted-Gene Network for Attachment of the Social Brain

Three lines of evidence (1) demonstrations that imprinted-gene expression and coexpression centrally mediate prenatal and postnatal growth, (2) diverse forms of data on imprinted-gene expression effects in the brain, and (3) expectations from theory that mother–offspring interactions should involve genomic conflicts, lead to the prediction that a brain-specific imprinted-gene coexpression network should exist, and should modulate aspects of human mother–child attachment. The presence of such a network in humans was evaluated by testing statistically for differentially frequent coexpression of brain-expressed imprinted genes, compared to expression of imprinted genes with other genes independent of imprinting status. For each of the 64 verified human imprinted genes (http://igc.otago.ac.nz/table.html), a set of highly coexpressed genes were generated using the Gemma coexpression database (http://www.chibi.ubc.ca/Gemma/searchCoexpression.html). These sets of coexpressed genes were combined to generate a list of genes that were highly coexpressed with four or more imprinted genes. The list comprised 188 genes in all, of which approximately 0.6% (64/~10,000 total brain-expressed genes)

should be imprinted under a null model of coexpression random with regard to imprinting status. Six (3.1%) of the 188 coexpressed genes were imprinted, an approximate fivefold excess ($P < 0.001$). The highly coexpressed imprinted genes included *PEG3* and *NDN*, two genes that mediate development of oxytocin-secreting neurons (Davies et al. 2008a, b); *NDN* may also be involved in the risk of schizophrenia (Le-Niculescu et al. 2007), and both *PEG3* and *NDN* are members of a network of genes differentially coexpressed in the prefrontal cortex of schizophrenics (module 16 of Torkamani et al. 2010). Additional relatively highly coexpressed genes in the network included three genes with genetic-association or copy-number variation links to both autism and schizophrenia, *CNTNAP2*, *NRXN1*, and *BOLA2* (reviewed in Crespi et al. 2010; coexpressed with 6, 7, and 11 imprinted genes, respectively). Additional data implicating this network, and imprinting effects, in autism and schizophrenia include (1) the presence of parent-of-origin effects for the associations of *CNTNAP2* with autism (Arking et al. 2008) and for the speech-language associated gene *FOXP2* (Feuk et al. 2006) that interacts directly with *CNTNAP2* (Vernes et al. 2008), and (2) colocalization of the imprinted, schizophrenia-associated gene *LRRTM1* (Francks et al. 2007) with *NRXN1* at glutamatergic synapses (Brose 2009).

This analysis requires replication using other datasets, and more detailed bioinformatic dissection of coexpression patterns, but it provides preliminary evidence of interactions between brain-expressed imprinted genes that mediate aspects of mother–offspring interaction and social-brain disorders. The existence and structure of brain-specific imprinted-gene network should have important implications for pharmacological interventions; for example, of the 11 genes most highly altered in expression from treatment of mice with the mood-stabilizer drug valproic acid (Chetcuti et al. 2006), two are imprinted (*Peg3* and *Sfmbt2*), one is predicted to be imprinted (*Zic1*)(Luedi et al. 2007), one interacts directly with the imprinted gene *Wt1* (*Par-4*)(Richard et al. 2001), and one (*Kcna1*) interacts directly with *Cntnap2* (Strauss et al. 2006). Such a striking concentration of imprinted-gene related expression changes is consistent with the molecular function of valproic acid as a histone deacetylase inhibitor, an agent that exhibits differential epigenetic effects on imprinted genes (e.g., Baqir and Smith 2006); moreover, valproic acid during human or rodent pregnancy is a highly penetrant cause of autism in offspring (Rinaldi et al. 2008; Dufour-Rainfray et al. 2010), and valproic acid partially restores levels of MeCP2 in a mouse model of Rett syndrome (Vecsler et al. 2010). Might such epigenetic treatments exert their effects though canalizing or decanalizing neurological-pathway functions of interacting imprinted genes?

## 8.10 Conclusions

Understanding human social development requires integration of theory and data from diverse, highly specialized, disciplines, including genetics, development, neuroscience, psychology, and psychiatry. Conrad Waddington's still-nascent

science of epigenetics serves as a conceptual and experimental platform to connect social-environmental variation with the gene-expression patterns that drive development, as genes and family, especially the mother, jointly sculpt developing brains. Like other traits, epigenetic landscapes of social development have evolved, subject to the conflicts and confluences of genetic interest that form the cornerstone of modern evolutionary theory. Effects of parent–offspring conflicts, and the intragenomic conflicts of imprinting, are predicted from such theory to modulate normal development and to potentiate specific directions and forms of maladaptive trajectory, due to genetic, epigenetic, and social-environmental perturbations. As such, dovetailing of predictions from evolutionary genetics and epigenetics with the conceptual constructs and experimental tools of developmental psychology, neuroscience, and epigenetics should yield novel insights into the evolutionary underpinnings of the human social brain and its disorders, and the strategies of the genes involved.

**Acknowledgments** I am grateful to Christopher Badcock, David Haig, Sarah Hrdy, and Felicity Larson for comments, and Paul Pavlidis for advice.

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# Chapter 9
# Genomic Imprinting Effects on Brain and Behavior: Future Directions

**Anthony R. Isles and Lawrence S. Wilkinson**

**Abstract** Studies over the last 15 years have indicated that genomic imprinting is important for brain function. However, much of the focus has been on the role that imprinted genes play in mediating fetal and early postnatal growth, and maternal behavior. Nevertheless, there is now a growing body of evidence to suggest that many imprinted genes are expressed in many different areas of the adult brain. Moreover, these genes also influence a wide range of behavior and aspects of cognition. Here, we provide an overview of these data and give pointers to interesting new aspects of imprinted gene function in the brain. We also discuss how genomic imprinting may have evolved in the human brain and the extent to which imprinted genes impact on mental illness.

**Keywords** Epigenetics · Evolution · Imprinted genes · Monoamines · Neurodevelopment

## 9.1 Introduction

Unlike the majority of genes where there is equivalent expression from both inherited copies of a gene (alleles), imprinted genes are subjected to epigenetic modifications during germ cell development that lead to paternal and maternal alleles having different levels of activity. This means that despite the presence of a maternal and paternal allele in the DNA, one of these parental alleles is essentially silenced and expression is monoallelic. This silencing is robust and stable across generations; for instance, the imprinted gene *Necdin* is always expressed only

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_9,
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from the paternal allele. Although imprinted gene representation in mammalian genomes is small (there are currently ~130 recognized imprinted genes in mice), they are absolutely crucial for normal development and function. Mouse embryos manipulated to have either only a paternal or only a maternal genome are malformed and die before mid-gestation (Barton et al. 1984; McGrath and Solter 1984). Since this early pioneering work, individual imprinted genes have been characterized and it is now clear that imprinted genes play a key role in three major areas: placental physiology, fetal, and preweaning growth (Constancia et al. 2004); energy metabolism (Smith et al. 2006); and brain development and behavior (Wilkinson et al. 2007).

### *9.1.1 Evolution of Genomic Imprinting*

Despite their apparent rarity in the genome, genes that are subjected to imprinting are (at least part of the time) effectively haploid, thus negating the apparent benefits of diploidy (increased genetic diversity and reduced exposure to deleterious alleles) (Goddard et al. 2005; Orr 1995). This is an evolutionary conundrum, and consequently, why imprinting has evolved is subject to much debate and several theories have been put forward (Curley et al. 2004; Hurst and McVean 1997, 1998; Moore and Haig 1991; Varmuza and Mann 1994; Wolf and Hager 2006). Two of these – the "intragenomic conflict" and "coadaptation" theories – provide an explanation for many of the widely observed physiological roles of imprinted genes (Keverne and Curley 2008; Moore and Haig 1991; Wolf and Hager 2006).

The intragenomic conflict theory, which was developed with kinship ideas in mind and is an extension of the classic "parent–offspring" conflict (Trivers 1974), suggests that where asymmetries of relatedness between maternal and paternal genes exist, there will be a conflict of interests between parental genomes over certain aspects of physiology (Haig 1997). These asymmetries of relatedness occur in developing fetuses as a consequence of multiple paternities, either within or between pregnancies. Consequently, maternally derived genes are always shared between siblings, but this is not necessarily true for paternal genes. Within an offspring, paternal genes would be predicted to attempt to increase resources from the mother, whereas maternally derived genes would limit any effect as this would not maximize the mother's (and therefore the maternal genes) overall reproductive output (Moore and Haig 1991). This imbalance leads to an "arms race" involving expression levels of maternal and paternal copies of a gene, ultimately resulting in the silencing of one parental allele. This intragenomic conflict can also be seen at a physiological level, as illustrated in the mouse by the *Igf2–Igf2r* system. The insulin-like growth factor type 2 (*Igf2*) is a paternally expressed growth enhancer, whereas the insulin-like growth factor type 2 receptor (*Igf2r*) is a maternally expressed growth inhibitor that binds Igf2 and targets it for degradation (Haig and Graham 1991).

By contrast, the coadaptation theory suggests that genomic imprinting occurs in mammals as a means to coordinate the evolution of provisioning of offspring pre- and postnatally. In this context, the monoallelic expression of imprinted genes is a means by which their "evolvability" is accelerated (Keverne and Curley 2008). This is because haploid expression, while increasing exposure to deleterious alleles, also has the advantage over diploid expression of rapid fixation of an advantageous trait in a population (Greig and Travisano 2003); although this effect is presumably lessened by the fact that monoallelic expression results in higher than average recombination rates (Necsulea et al. 2009). It is thought that the development of a placenta and maternal care in mammals is an area of function where such rapid fixation of any selective advantage is important, leading to the evolution of imprinted genes influencing these aspects of physiology (Keverne and Curley 2008; Wolf and Hager 2006).

This idea was originally developed in light of the identification of two separate paternally expressed genes in the mouse (*Paternally expressed 1* and *Paternally expressed 3*), both of which were found to effect placental function and fetal growth, and maternal care in adult females (Lefebvre et al. 1998; Li et al. 1999). Further analysis also revealed that *Peg3* was not only important for mothers providing postnatal care and feeding, but was also involved in the neonates' ability to suckle (Curley et al. 2004). The idea proposed was that the high turnover of the genes down the paternal line would provide the platform for the rapid coadaptation of these in utero and postnatal provisioning and care (Keverne and Curley 2008). Recently, a similar argument, based on the adaptive integration of offspring and maternal genomes, has been made for the evolution of maternally expressed imprinted genes (Wolf and Hager 2006).

Both of these theories provide an explanation for a large proportion of the observed physiological effects of imprinted gene; although no one theory can account for all occurrences. However, with regards to the adult brain, at present, the coadaptation theory offers only an explanation for the role of imprinted genes in the hypothalamus controlling maternal behavior (something the intragenomic conflict theory struggles with) and yet imprinted genes are now known to be expressed in many brain regions and influence many other behaviors.

## 9.2 Imprinted Gene Expression in the Brain

### *9.2.1 Early Findings*

Seminal mouse studies in the mid 1990s revealed that imprinted genes are likely to contribute significantly to brain development, and also indicated potentially dissociable (and possibly antagonistic) influences of paternally and maternally expressed genes on this process. Briefly, chimeric mice were created, which contained either a mixture of androgenetic (AG) (containing two paternal genomes

but no maternal genome) and normal cells, or a mixture of parthenogenetic/gynogenetic (PG/GG) (containing two maternal genomes but no paternal genome) and normal cells. "PG/GG chimeras" displayed relatively large brain:body ratios, whereas "AG chimeras" displayed relatively small brain:body size ratios, implying that one or more imprinted genes have profound effects on brain size (Allen et al. 1995; Keverne et al. 1996a). Specifically, the data seem to indicate that the overall effect of maternally expressed genes is to enhance brain size, whereas the combined effect of paternally expressed genes is to limit brain growth. More interesting perhaps, was the fact that the distribution of the PG/GG and AG cells in the two types of chimera was reciprocal, with PG/GG cells contributing mainly to the neocortex, and AG cells contributing more to the hypothalamic, septal, and preoptic areas. This provided the first suggestion that imprinted genes of different parental origins may have differential "interests" with regards to the brain. These effects may represent either the combined effects of many paternally and maternally expressed imprinted genes, or the actions of one or two imprinted genes of major effect. If the former is the case, we may expect maternally expressed imprinted genes to be disproportionately expressed in neocortical regions and paternally expressed imprinted genes to be disproportionately expressed in hypothalamic and septal regions; this is not exactly the case (Davies et al. 2005b) and the answer is probably somewhere in between. In reality, the distribution of AG and PG/GG cells in the two types of chimera probably points to where the interests of maternal- and paternal-imprinted genes in the brain lie when unfettered by the action of opposing parental alleles.

### *9.2.2 New Genes*

Since those pioneering experiments, many imprinted genes (~90%) have been shown to be brain expressed. We have published a number of reviews detailing the characteristics of imprinted genes in the brain (Davies et al. 2005b) and also have produced a freely accessible online database of imprinted genes expressed in the brain (Davies et al. 2007). However, a number of new brain-expressed imprinted genes have recently been identified, partly due to the development of new screening methods (Schulz et al. 2006). Many of these have been identified in humans, often via the study of neurodevelopmental (Francks et al. 2003, 2007; Wawrzik et al. 2010) or psychiatric disease (Francks et al. 2003, 2007); the possible importance of this is discussed later.

One criticism that is often leveled at the imprinting community is that despite their developmental importance, there is a relative paucity of imprinted genes in the mammalian genome. Although a number of imprinted microRNAs have recently been identified, the overall number of imprinted gene still remains low, being around 130 (Beechey et al. 2003). In light of this criticism, it will be interesting to see the impact of more focused association studies aimed at identifying traits and diseases showing parent of origin effects (Kong et al. 2009). However, probably

more important to the discovery of novel imprinted genes is the development of next-generation sequencing methods (Mardis 2008). It has been known for some time that a number of imprinted genes, particularly in the brain, show complex patterns of epigenetic regulation (Albrecht et al. 1997; Yamasaki et al. 2003). The result of this is that some genes are only imprinted in certain tissues (or brain regions) and/or certain developmental time points, while being expressed in a biallelic manner in others (Davies et al. 2005b). In the past, this subtle regulation would have been a hindrance to the identification of new imprinted genes. However, next-generation sequencing technologies are capable of producing tens of millions of sequence reads from very little input material. This is expected to revolutionize how we analyze gene expression and would, for instance, allow the quantification of allelic expression from discrete tissue samples.

### *9.2.3 Filling in the Details*

In addition to identifying new candidates, interest in the physiological function of imprinted genes continues to grow within the imprinting community. This has led to the gaps in our knowledge of the expression patterns and neural function of previously known brain-expressed imprinted genes being filled. In particular, the neural and biochemical role of a number of imprinted genes of relevance to disease, such as *Ube3a* (Greer et al. 2010; van Woerden et al. 2007; Yashiro et al. 2009) and *Necdin* (Kuwajima et al. 2006; Kuwako et al. 2005; Zanella et al. 2008), have been studied in some detail.

One striking recent finding is the relative importance of two paternally expressed brain-specific small nucleolar (sno)RNA molecules, *snord115* and *snord116* (formerly known as *mbii-52* and *mbii-85*, respectively). snoRNAs are noncoding and thought to negatively regulate posttranscriptional modification (alternate splicing and RNA editing) of other pre-RNA species (Kishore and Stamm 2006). *snord116* is strongly expressed in the hypothalamus, suggesting a role in regulating feeding and metabolism. Although its target genes have not been identified, a knockout of *snord116* has demonstrated that its loss has important consequences for early postnatal survival and homeostasis and therefore makes it a key candidate for the imprinting disorder Prader–Willi syndrome (PWS) (Ding et al. 2008). More recently, a study of a clinical case has confirmed that loss of *SNORD116* is the main (but probably not sole – Peters 2008) contributor to the phenotype of the imprinting disorder PWS (Sahoo et al. 2008).

The second snoRNA, *SNORD115*, is also part of the PWS-imprinting cluster, but unlike *SNORD116*, its targets and mode of action are much better understood. *SNORD115* has a clear complimentary region for serotonin 2C receptor (*5htr2c*) pre-RNA exon Vb, a region that is subjected to both RNA editing and alternate splicing (Cavaille et al. 2000) and shows a reasonable overlap in expression with *5htr2c* found in cortical regions, the hippocampus, striatum and hypothalamus (Vitali et al. 2005). Loss of *snord115* in a mouse model for PWS results in increased

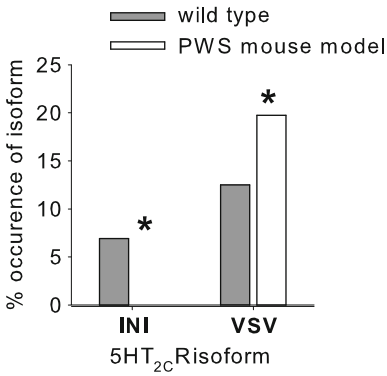


**Fig. 9.1** Predicted pattern of two key serotonin 2C receptor protein isoforms in PWS mouse model brain. Data are based on the pattern of RNA editing as revealed by sequencing analysis (Doe et al. 2009). The INI isoform results as a consequence of no RNA editing, and is completely absent in PWS mouse brain. The VSV is the most abundant in brain and results as a consequence of editing at most of the five potential sites in the *5htr2c* pre-RNA. This is significantly increased in PWS mouse brain

levels of RNA editing across the five edited sites in exon Vb of the *5htr2c* pre-RNA. Such adenosine-to-inosine editing has consequences for the receptor protein function as inosine behaves like a guanine in translation, resulting in altered amino acid sequence. Bioinformatic analysis indicates that the pattern of serotonin receptor protein isoforms in the PWS mouse model brain is significantly altered, with a decrease in functional, and an increase in less function variants (Fig. 9.1).

#### 9.2.3.1 Genomic Imprinting and the Monoamine System

More detailed analysis of imprinted genes expressed in the brain has thrown up the beginnings of an interesting pattern relating to monoamine system. A number of imprinted genes are now known to influence the function of one or more of the members of the monoamines (see Table 9.1). Some genes, such as *Necdin*, may be having general neurodevelopmental effects (Zanella et al. 2008) or they are not centrally important to monoamine function like *Ddc* (Menheniott et al. 2008). However, many more others, such as *Th*, *5htr2a*, and the snoRNA *snord115* described above, are directly linked to the efficacy of monoamine function in the brain.

Most interesting of all perhaps is the overlap in expression of *Dlk1*, *Nesp*, and *Grb10* in the brain (Jensen et al. 2001; Plagge et al. 2005). All three of these genes are expressed in discrete regions of the brain, but show important overlap in the locus ceoreleus and raphe nuclei. These two midbrain regions are important for noradrenergic and serotonergic projections to forebrain. Furthermore, *Dlk1* and *Grb10* are also expressed in the dopaminergic ventral tegmental area and the substantia nigra. This striking overlap in expression ties all three of these imprinted

**Table 9.1** Imprinted gene involvement in the monoamine system

| Gene(s) | Parental expression | Relation to monoamine system | References |
|---|---|---|---|
| *Dlk1* | Paternal | Expressed in areas with monoaminergic projections to forebrain. Involved in dopamine neuron differentiation | Jacobs et al. (2009) |
| *Nesp* | Maternal | Expressed in areas with monoaminergic projections to forebrain, including locus coeruleus and dorsal raphe nucleus | Plagge et al. (2005) |
| *Grb10* | Paternal | Expressed in areas with monoaminergic projections to forebrain, including locus coeruleus, dorsal raphe nucleus, and striatum | |
| *Th* | Maternal | Tyrosine hydroxylase; upstream enzyme in the production of dopamine and noradrenaline important in many aspects of monamine mediated behavior | Palmiter (2008) |
| *CDKN1C* | Maternal | Cooperates with Nurr1 in directing dopaminergic neuron differentiation | Joseph et al. (2003) |
| *H19-IGF2* | Maternal and paternal | Many of the genes in this interval are highly expressed in dopaminergic progenitor cells | Freed et al. (2008) |
| *5HTR2A* | Maternal | Serotonin receptor 2A | Bunzel et al. (1998), Kato et al. (1996) |
| *Magel2* | Paternal | Mice lacking *magel2* show altered serotonin metabolism in the cortex and reduced dopamine in hypothalamus | Mercer et al. (2009) |
| *snord115* | Paternal | Regulates posttranscriptional modification of the serotonin 2C receptor | Doe et al. (2009) |
| *Ddc* | Paternal | Dopa decarboxylase; imprinted in heart | Menheniott et al. (2008) |
| *Necdin* | Paternal | Modulation of seronergic innervation of respiratory system | Zanella et al. (2008) |

genes (two paternally and one maternally expressed) to key areas in monoaminergic function. Nevertheless, thus far, only *Dlk1* has been explicitly shown to be important in dopamine neuron differentiation (Christophersen et al. 2007; Jacobs et al. 2009; Jensen et al. 2001). Although loss of *Nesp* and *Grb10* results in behavioral changes (Plagge et al. 2005), it is not clear as yet whether these behavioral changes are due to specific alterations in monoamines.

## 9.3 Behavior

Much of the original work on imprinted genes in the brain has centered on mother–pup interactions. Deficits were seen in two mouse models, null for *Peg1* and *Peg3*. In addition to mothering deficits in the adults, *Peg3* null pups also show poor postnatal development, such as soliciting suckling and homeostasis

(Curley et al. 2004). This pattern of physiological deficit has been seen in a number of other imprinted gene mutants (Plagge et al. 2004; Skryabin et al. 2007) and is also seen in the children with PWS (Haig and Wharton 2003). Whether these early life deficits have knock-on effects for adult behavior is a distinct possibility (Francis et al. 1999) and has been suggested as a possible cause of the psychiatric problems seen in genomic imprinting syndromes such as PWS (Isles and Holland 2005).

However, just as it has become increasingly recognized that imprinted genes are expressed in the adult brain, it has become clear that many may also directly influence other adult behaviors. Apart from the *Peg1* and *Peg3* studies, much work has focused on those imprinted genes within the PWS cluster. Many of these genes are expressed primarily in the hypothalamus, and the behavioral studies reflect this. As such, in addition to maternal behavior, we now know that imprinted genes influence sexual behavior (Swaney et al. 2007), circadian rhythms (Kozlov et al. 2007), feeding, and homeostasis (Ding et al. 2008). Nevertheless, as we have outlined earlier, imprinted genes are also found in many other brain regions, including the prefrontal cortex, hippocampus, striatum, and areas of importance for mediating neurotransmitter innervations of cortical regions.

### *9.3.1 Attention, Impulsivity, and Behavioral Flexibility*

There is a growing body of work demonstrating that imprinted genes are also important for aspects of cognition. This includes some molecules with well-established roles in learning and memory such as the ubiquitin protein ligase E3A encoded by the maternally expressed *Ube3a* gene (Jiang et al. 1998), and the paternally expressed Ras signaling molecule *Grf1* (Brambilla et al. 1997; Giese et al. 2001).

Given increasing prominence of imprinted genes in monoamine function, it is not unsurprising that genomic imprinting has also been found to impact on other discrete aspects of cognition. Recently, independent studies of people with PWS and a mouse model for PWS have demonstrated deficits in executive function. Typically, individuals with PWS have mild learning difficulties as indicated by a general reduction in IQ scores (Whittington et al. 2004). Although explicit psychological testing is limited, it is known that there is a reduced ability in attention and visuoperceptual organization in the PWS subjects when compared with the normal population (Jauregi et al. 2007) and even when corrected for general intellectual ability (Woodcock et al. 2009). The work on the animal model for PWS used the 5-choice serial reaction time task (5-CSRTT), which is a form of continuous performance task, assays components of executive functioning, taxing aspects of visuospatial attention and inhibitory control, and has been extensively characterized as a valid cross-species behavioral test (Humby et al. 1999; Robbins 2002). The findings from the 5-CSRTT point to reduced attentional capabilities in the PWS mice. Although the animals could acquire the task to our criteria levels, their baseline performance was impaired across three main measures: with PWS animals showing decreased

accuracy, increased omissions, and slower correct reaction times. Moreover, under a task manipulation considered to tax attentional functioning (reduced stimulus duration), the PWS mice displayed increased deficits relative to controls. These animal data are coupled with clinical findings demonstrate a specific contribution of the paternally expressed genes in the PWS interval to attention.

Another aspect of cognition upon which genomic imprinting impacts is response control, or the processes that are involved in what is more generally known as impulsivity and behavioral flexibility. In fact, genomic imprinting has been linked with impulsive behavior previously, though not explicitly (Higley and Linnoila 1997; Higley et al. 1993). However, recent work has now unambiguously linked genomic imprinting with different aspects of response control. This includes the maternally expressed Nesp55, which when deleted reduces the willingness of mice to explore a novel environment (Plagge et al. 2005); and studies of mice monosomic for the X chromosome, which have shown that lack of a paternal X gives rise to deficits in behavioral flexibility on a reversal learning task (Davies et al. 2005a).

Nevertheless, thus far, only one study has really explored the impact of an imprinted gene on the neural substrates of impulsive behavior. As outlined earlier, the snoRNA *snord115* plays an important role in the regulation of posttranscriptional modification of the *5htr2c* pre-RNA. Consequently, in a mouse model of PWS that lacks expression *snord115*, there is an increase in abundance of the less functional isoforms of serotonin 2C receptor ($5HT_{2C}R$) (Fig. 9.1). The $5HT_{2C}R$ plays an important role in modulating aspects of response control, which when measured in the 5-CSRTT is due to its specific effects on dopamine release in the nucleus accumbens (Robinson et al. 2008). When behavior of mice lacking *snord115* in the 5-CSRTT is probed with $5HT_{2C}R$ selective drugs, an enhanced increase in impulsivity is seen relative to controls (Doe et al. 2009). This effect is specific, as drugs for $5HT_{2A}R$ have no differential effect. This work indicates that the paternally expressed *snord115* is an important factor in mediating $5HT_{2C}R$ controlled impulsivity.

## 9.4 Evolution of Imprinted Genes in the Human Brain

The development of genomic imprinting in animals (true genomic imprinting has also evolved independently in plants – Kinoshita et al. 1999) seems to be linked with the evolution of mammals (Renfree et al. 2009). This suggests that key aspects of being mammalian, namely in utero development, a placenta, and postnatal parental care, were the original driving forces in the evolution of imprinting; the extensive role of imprinted genes in these functions (Constancia et al. 2004) would point to this being true. However, recent data from parental expression and/or epigenetic marking studies of imprinted genes known to influence in utero growth in rodents indicate that many have lost their monoallelic status in humans (Isles 2009). This would imply that the selective pressure on maintenance of the imprinting status of genes in the placenta and developing fetus is reduced or absent in humans. Thus far, two

explanations for this have been suggested, namely the fact that human reproductive strategies have change (Monk et al. 2006) and that increased resourcing of the later stage fetus is not desirable due to our bipedalism (Isles 2009).

Nevertheless, while the importance of imprinted genes in the placenta and prenatal growth in humans is diminished, the opposite may be true of imprinted genes in the brain. Certainly, a key area where imprinted genes exert an influence is the early postnatal period. Several studies in mice (Curley et al. 2004; Ding et al. 2005; Plagge et al. 2004; Shiura et al. 2005; Skryabin et al. 2007) and human disorders (Haig and Wharton 2003) have indicated that in addition to in utero, imprinted genes can also impact upon nutrient acquisition and growth while the offspring are still dependent on their mother for feeding. Indeed, the rapid increase in body weight and size over the first year of life (Bluestone 2005), coupled with the extended dependence of human offspring for nutritional support (Sellen 2007), make postnatal feeding an important arena for imprinted genes (Haig 2010). Moreover, the acquisition of "social resources" (in addition to nutritional resources) from the mother (Horsler and Oliver 2006; Isles and Holland 2005; Oliver et al. 2007) during this crucial neurodevelopmental period may also be an important substrate for the evolution of genomic imprinting; these ideas are developed further elsewhere within this book (Bernard Crespi 2010). However, it is increasingly clear that some imprinted genes have an important role in the brain beyond this mother–offspring interaction. Furthermore, although much of the work on these adult brain expressed imprinted genes has been carried out in rodents, there is a suggestion that this role may be increasingly important in humans.

Although it is highly likely that the driving force behind the evolution of genomic imprinting was the development of an extended pregnancy and parental care, once established other selective pressures may have led genomic imprinting to be co-opted to act on other functions, such as brain and behavior. There is some evidence for this; a number of brain expressed imprinted genes such as *Neuronatin* (Evans et al. 2005) and Nesp (Gavin Kelsey pers. comm.) appear only later in the Eutherian mammalian lineages. Interestingly, there appears to be even more enrichment in humans, with the genes *LRRTM1*, *C15ORF2*, and *5HTR2A* only occurring or being imprinted in the human brain (Francks et al. 2003, 2007; Wawrzik et al. 2010).

Although these data are limited, they do provide a tantalizing suggestion that genomic imprinting in the brains of humans may be particularly important. Obviously, of direct relevance to this question is the analysis of imprinted genes in other species of primates; unfortunately much of the data we have simply allows a comparison between mouse and human. However, two separate studies of brain-expressed imprinted genes have directly addressed the question of whether these genes are particularly important for humans. As mentioned earlier, paternally expressed *C15ORF2* is only found in man, but more detailed analysis has also pointed to the fact that it has been positively selected through human evolution (Wawrzik et al. 2010). Similarly, the maternally expressed *Klf14* has also undergone accelerated evolution in humans (Parker-Katiraee et al. 2007). However, this gene is also present (and imprinted) in mice, suggesting that the positive selection is very much related to some key changes in the human lineage.

What selective pressures have led to this is not clear at present. One idea links back to the original work with mouse chimeras demonstrating differential distribution of PG/GG (maternal genomes only) and AG (paternal genome only) cells in the brain (Keverne et al. 1996b). The distinct brain regions to which PG/GG and AG cells contribute have also evolved differentially in primates. While the frontal cortex and striatal areas have expanded relative to the rest of the brain, the hypothalamus and septum have contracted in size. The forebrain areas that have expanded are thought to have done so due to the selective pressure of living in social groups and all complexities of social behavior which that entails. In the majority of primate societies, the maintenance of social cohesion and group continuity over successive generations is dependent on the matriline; these are also in the "interests" of the maternal genome, which is more likely to be shared among the members of the group. It seems more than coincidental that the areas of brain expansion required for living in social groups are also those to which the maternal genome makes a substantial developmental contribution. In humans, social behavior is taken to another level and so it may be the case that the importance of imprinted genes in the brain has also increased.

### *9.4.1 Consequences of Genomic Imprinting for Mental Illness*

Whether or not they are adaptive, the increased numbers of imprinted genes expressed in the human brain may still be important contributors to abnormal brain function, particularly those with pleiotropic effects where any secondary phenotypes may be suboptimal as a result of the intragenomic conflict (Wilkins 2010). Indeed, there is an increasing evidence to suggest that this is the case not only from imprinting syndromes, such as PWS and Angelman syndrome, but also from other genome-wide studies of mental illness.

The contribution of genomic imprinting to disease may actually be amplified as the tight regulation of imprinted genes means that in addition to classic coding changes, they are also particularly susceptible to any mutations that cause alterations in expression levels. This added vulnerability may occur in two ways: either incorrect epigenetic regulation of the genes or changes in the dosage. The former has not been well explored, although the increased incidence of imprinting syndromes in individuals born as a result assisted reproductive technologies hints at the potential problems (Isles and Wilkinson 2008). The consequences of alterations in gene dosage have recently been revealed in a number of genome-wide studies linking Copy Number Variations (CNVs) with neuropsychiatric problems. Among those areas identified as important contributors to mental illness is the PWS-imprinted gene interval, 15q11–q13. This region has been linked in a number of independent studies to both schizophrenia (Kirov et al. 2009; Stefansson et al. 2008) and autism (Glessner et al. 2009; Sebat et al. 2007), with maternal duplications being particularly problematic (Cook et al. 1997; Schroer et al. 1998).

**Note added in proof** Some new and exciting results have been published since this article was submitted. Firstly, next-generation sequencing techniques have been used to examine the parent-of-origin specific expression bias in the brains of reciprocal cross F1 mouse sub-strains. These studies revealed existence of approximately 1300 genes showing a parent-of-origin specific bias in their expression (Gregg *et al*. *Science* **329**:643–8 and *Science* **329**:682–5). Moreover, these studies identified the hypothalamus and monoaminergic regions (noradrenergic locus coeruleus, serotonergic dorsal raphe nucleus and dopaminergic Nucleus accumbens and Ventral tegmental area) as being key hotspots for expression of these candidate imprinted genes. Supporting this idea was a recent paper examining the brain expression of the imprinted gene *Grb10*. This too was expressed in these specific brain regions and also appeared to co-localise with the dopamine transporter (Garfield *et al*. *Nature* **469**:534–38).

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# Chapter 10
# Epigenetic Influence of the Social Environment

**Frances A. Champagne and James P. Curley**

**Abstract** Social experiences occurring during infancy have been demonstrated to exert persistent effects on neurobiological and behavioral outcomes. This social modulation of the developing brain has been observed in humans and animal models of abuse, neglect, and variation in parental style. Although the mechanisms through which these effects are achieved likely involve diverse cellular and molecular pathways, there is emerging evidence supporting the hypothesis that epigenetic changes, such as DNA methylation and histone modifications, may mediate the effects of early life variations in the social interactions between mothers and infants. Moreover, there may be plasticity within these epigenetic pathways at later developmental time points, such that the social experiences of juveniles and adults may also induce epigenetic change. These findings have implications for behavioral variation observed both within and across generations and highlight the dynamic interactions occurring between genes and environments during the course of development.

**Keywords** Abuse · Epigenetic · Maternal · Neglect · Neurodevelopment · Parenting · Transgenerational

## 10.1 Introduction

The quality of the social environment can have a significant impact on physiology, neurobiology, and behavior. There is growing evidence from epidemiological studies in humans for the persistent effects of the early life experience of abuse, neglect, and variations in parenting style, which suggest that multiple neural

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_10,
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systems may be subjected to modulation via the social context of development. Further, explorations of the mechanisms through which these effects are achieved have focused on experimental paradigms involving primate and rodent models of variation in the social environment, and in particular, disruption of postnatal mother–infant interactions. Although multiple molecular and cellular pathways are implicated in mediating the link between early life experiences and long-term changes in phenotype, recent evidence has highlighted the role of epigenetic mechanisms, such as DNA methylation and posttranslational modifications to histone proteins within the nucleosome. Although shifts in DNA methylation were once thought to be restricted to early embryonic development, studies of nutritional (Lillycrop et al. 2007, 2008), chemical (Onishchenko et al. 2008), and a broad range of environmental exposures (Mueller and Bale 2008; Roth et al. 2009; Weaver et al. 2004) occurring during pre- and postnatal development have implicated epigenetic regulation of gene expression as a critical target of experience-dependent change. In this chapter, we describe the experimental approaches that have been used to explore the long-term effects of early life social experiences; highlight evidence from humans, primates, and rodents for social modulation of the brain; and illustrate the role of epigenetic mechanisms in maintaining the effects of the social environment. Although plasticity in development is typically associated with the perinatal period, there is continued social modulation of gene expression and behavior among juveniles and adults and this plasticity may likewise involve epigenetic modifications. Moreover, the impact of the social environment may not be restricted to within-generation effects and may lead to the transgenerational inheritance of phenotypic variation that involves experience-dependent changes in DNA methylation (Champagne 2008). These findings suggest that an exploration of epigenetic mechanisms may advance our understanding of the complex and dynamic interplay between the genome and the environment.

### *10.1.1 From Epidemiology to the Laboratory: Strategies for Studying Early Life Social Influences*

Epidemiological and longitudinal studies have provided significant support for the hypothesis that the quality of the early life social environment may shape developmental trajectories leading to either risk or resilience to later life psychiatric disorder. In humans, neglect and abuse have been demonstrated to reduce cognitive performance and impair social development (Trickett and McBride-Chang 1995) and is associated with a fourfold increase in personality disorders (Johnson et al. 1999). The severe neglect experienced by institutionalized infants, most recently explored among Romanian orphans, further demonstrates the persistent effects of these experiences. Delays in growth, social, and cognitive development observed in Romanian orphans are associated with later life impairments in attachment, heightened inattention, and increased autistic-like behaviors (Beckett et al. 2002;

MacLean 2003; O'Connor and Rutter 2000; O'Connor et al. 2000; Rutter and O'Connor 2004). Although it is difficult to identify the particular aspect of the neglectful or abusive childhood experience which contributes to this outcome, disruption to the mother–infant relationship, consisting of both physical and emotional contact, associated with these conditions is thought to be critical. Variations in the attachment relationship between mother and infant have been associated with either resilience to psychological distress or increased incidence of psychopathology (Sroufe et al. 1999; Sroufe 2005). Secure attachment, typically assessed by the Strange Situation Task (in which infants' response to separation followed by reintroduction of the mother is measured) is associated with increased social competence and cognitive performance, whereas disorganized attachment patterns predict increased rates of borderline personality disorder, dissociation, and self-harm in adulthood (Carlson 1998; Carlson et al. 2009). Studies using a retrospective assessment of the quality of the mother–infant relationship, such as the Parental Bonding Index (PBI), suggest that low levels of maternal care combined with controlling–overprotective parenting are a significant predictor of adult depression (Parker et al. 1979; Parker 1993). Overall, these studies indicate that disruption to the early social environment, particularly the interactions between mother and infant, can have sustained effects on numerous biobehavioral outcomes in adulthood.

Our understanding of the mechanisms linking these early life experiences to adult outcomes has come from the development of animal models, which incorporate aspects of neglect, abuse, or variation in the quality of the mother–infant interactions' that are evident in human longitudinal studies (see Fig. 10.1). The classic studies of Harry Harlow on the development of rhesus macaques exposed to maternal deprivation provide evidence that the absence of mother–infant interactions during the early phases of development can induce disruptions to social play, hyperactivity, and sensitivity to stressors (Harlow et al. 1965; Seay and Harlow 1965; Suomi et al. 1971). The importance of mother–infant contact is further

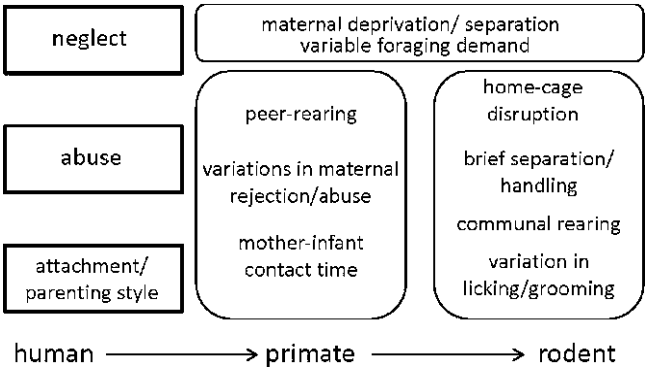


**Fig. 10.1** Summary of paradigms used to study social modulation of brain and behavior during postnatal development in humans, primates, and rodents

demonstrated by the persistence of abnormalities in development that emerge in response to peer-rearing (Suomi 1991), where infants have social contact with the peers but not with the mother. In primates, variable foraging demand can also be used to disrupt the quality and quantity of mother–infant interactions (Coplan et al. 2006). When the duration of time that is needed to locate and retrieve sufficient amounts of food is inconsistent across weeks, the sensitivity of mothers to infant cues is disrupted, and consequently, offspring exhibit elevated levels of anxiety-like behavior and are less social in adulthood (Coplan et al. 1995, 2005; Gorman et al. 2002). These variations in mother–infant interaction are also observed to occur naturally among colonies of rhesus and pigtail macaques. Maternal abuse in the form of dragging and stepping-on infants occurs in isolation-reared and group-housed macaques at a frequency of 2–10% (Berman 1990; Carroll and Maestripieri 1998; Maestripieri 1998). Abused infants show a delayed onset in social play and are hyperaggressive in novel environments (McCormack et al. 2006). Abusive mothers also engage in high levels of maternal rejection, where infant attempts to make contact with the mother are rejected, and the experience of high levels of this parenting style may be associated with neurobiological outcomes associated with infant abuse (as discussed in the next section). High levels of mother–infant contact (characteristic of an over-protective parenting style) have also been observed in non-human primates and are associated with decreased exploration of a novel environment when infants are juveniles (Fairbanks and McGuire 1988). Thus, both experimental manipulation of the early social environment and observational studies of naturally occurring variations in mother–infant interactions can be used to explore the persistent effects of social experiences.

Although primate studies provide a useful model for exploring the effects of neglect, abuse, and variations in maternal behavior that may shape the developing brain and behavior, our understanding of the mechanisms through which this is achieved have relied primarily on studies of laboratory rodents. Maternal neglect and deprivation can be experimentally induced by separating pups from the dam for extended periods of time, a manipulation referred to as maternal separation (Rosenfeld et al. 1992), or through rearing pups in complete isolation from the dam, referred to as artificial rearing (Hall 1975). In general, prolonged separation or deprivation from maternal contact induces heightened anxiety-like behaviors, reduced performances on learning and memory tasks, and decreased social behaviors in adulthood (Lehmann et al. 1999; Lovic and Fleming 2004). The duration of separation is an important modulator of this effect, and there is evidence that brief maternal separations (often referred to as "handling") can stimulate maternal behavior and attenuate the stress response (Levine 1957; Meaney et al. 1991). The effects of abusive caregiving can also be studied in rodents. Removal or disruption of the bedding material normally included in the cages of laboratory rats and mice can induce dams to engage in rough handling, stepping-on, and avoidance of pups (Roth and Sullivan 2005). This paradigm has been used primarily for studying the factors influencing attachment to abusive caregivers and may also be a useful approach for studying the effects of early life trauma. Extended periods of maternal separation can also be induced in the laboratory by imposing foraging demands, and evidence

from studies in mice suggests that a variable foraging demand on dams is associated with increased anxiety-like responses in male offspring (Bredy et al. 2007; Coutellier et al. 2009). Interestingly, studies of biparental voles indicate that removal of the father can have lasting effects on neurodevelopment (Ahern and Young 2009), though the benefits of multiple caregivers for offspring development is not limited to biparental species. In laboratory mice, communal rearing can be used to study the effects of increased social interactions during the postnatal period (Branchi 2009). In a communal nest, multiple postparturient females are housed together with their own litters or foster pups and the litters are combined and cared for as a group by the lactating dams. When compared with standard reared pups, offspring reared in communal nests are found to exhibit changes in anxiety-like and social behavior that are dependent on the age distribution of pups in the nest and conditions of the testing environment (Branchi and Alleva 2006; Branchi et al. 2009; Curley et al. 2009). This rearing paradigm has been demonstrated to ameliorate many of the behavioral deficits characteristic of the highly anxious BALB/c mouse strain (Curley et al. 2009), suggesting the modulating effect of social experiences on strain differences in behavior.

Individual variation in maternal styles that are observed in humans and primates are also exhibited by laboratory rodents and can likewise be associated with divergent developmental outcomes. In rats and mice, there are individual variations in several aspects of maternal behavior during the first week postpartum (Champagne et al. 2003a, 2007). In particular, there are stable between-dam variations in the frequency of pup licking/grooming (LG). One strategy for studying the long-term influence of mother–infant interactions is to characterize the LG behavior of a cohort of lactating females and compare outcome measures between offspring reared by Low or High LG dams [with Low or High being defined as 1 SD below or above the cohort average LG (Champagne et al. 2003a)]. This approach has been used successfully to study the origins of individual differences in stress response, response to novelty, learning and memory, and numerous indices of social/reproductive behavior (Meaney 2001). Variations in LG during the postnatal period can also be induced by gestational stress (Champagne and Meaney 2006; Moore and Power 1986), postparturient exposure to predator odor (McLeod et al. 2007), and various manipulations of the postnatal and juvenile rearing environment of the dams (Champagne and Meaney 2007; Lovic et al. 2001), with consequences for offspring development. Thus, plasticity in maternal behavior in response to environmental conditions is one route through which the quality of the environment can shape offspring physiology, brain, and behavior.

### *10.1.2 Social Modulation of the Developing Brain*

The rodent and primate models of neglect, abuse, and variations in mother–infant interaction described in the previous section have been used to explore the neurobiological impact of the social environment and have yielded target neural systems,

which have been subsequently explored in human cohorts. Disruption to the early life environment has been demonstrated to exert persistent effects on the hypothalamic–pituitary–adrenal (HPA) response to stress (for recent reviews see Korosi and Baram 2009; Lupien et al. 2009). Elevations in glucocorticoids associated with exposure to stress is achieved via release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus that acts on CRH receptors within the pituitary to trigger the release of adrenocorticotropin hormone (ACTH) and consequent release of glucocorticoids from the adrenal cortex. This HPA activity can be potentiated by neuropeptides such as vasopressin (AVP) and down-regulated through a negative feedback loop involving hippocampal glucocorticoid receptors (GR). In early development, social experiences that promote increased levels of mother–infant tactile stimulation generally lead to long-term reduction in HPA stress reactivity. Adult rats that were reared by High LG dams or exposed to postnatal handling have attenuated stress responsivity, reduced CRH mRNA expression in the PVN, and higher expression of GR in the hippocampus (Francis et al. 1999; Liu et al. 1997; Meaney and Aitken 1985; Meaney et al. 1985, 1989; Viau et al. 1993). In contrast, postnatal maternal separation induces increased stress reactivity associated with reduced GR expression in the hypothalamus and hippocampus, and regional changes in CRH receptor expression (Ladd et al. 2004; Plotsky and Meaney 1993). Thus, neural circuits involved in emotionality are susceptible to modulation in response to early life experiences, particularly those experiences affecting the frequency of mother–infant interactions.

The influence of the social environment is not limited to neuroendocrine pathways, which are primarily involved in regulating the stress response. However, it should be noted that most investigations of the neurobiological consequences of experimental manipulations of the early life environment use a target gene/neural system approach such that the specificity vs. breadth of the effects of particular manipulations are not well elucidated. Maternal separation is associated with increased dopamine (DA) in the striatum of mice (Ognibene et al. 2008). Moreover, compared with individuals who were handled during the first 2 weeks of life, adults who experienced postnatal maternal separation have increased dopamine D1 receptor binding levels in the nucleus accumbens (NAc) core and caudate putamen, increased D3 receptor mRNA in the NAc shell, and decreased levels of dopamine transporter (DAT – which uptakes DA from the synapse) (Brake et al. 2004). Maternally separated rat pups have decreased 5-HIAA and HVA (serotonin (5-HT) metabolites) levels in the amygdala and increased stress-induced 5-HT and 5-HIAA levels (Arborelius and Eklund 2007). Similar increases in 5-HT levels are found in the prefrontal cortex, hippocampus, and striatum of mice that are maternally separated during the first week postpartum (Ognibene et al. 2008). Handling-induced increases in gamma-aminobutyric acid (GABA) A central benzodiazepine (CBZ) receptors have been detected in the medial prefrontal cortex (mPFC), hippocampus, and amygdala (Bodnoff et al. 1987; Caldji et al. 2000; Weizman et al. 1999). Extended periods of maternal separation in rats have been reported to cause reductions in the expression of the hippocampal *N*-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor subunits (Bellinger et al. 2006; Pickering et al. 2006;

Roceri et al. 2002). Postnatal maternal separation has also generally been associated with elevated AVP immunoreactivity and mRNA in the PVN (Vazquez et al. 2006; Veenema et al. 2006; Veenema and Neumann 2007), though some studies only find this increase in subjects undergoing a subsequent exposure to stress (Veenema et al. 2006). Maternal separation leads to increased vasopressin V1A receptor (V1Ar) binding in the lateral septum (LS) of juvenile males (Lukas et al. 2010). Maternally separated male rats have lower oxytocin receptor (OTR) binding in the LS and caudate putamen, and higher OTR binding in the medial preoptic area (MPOA) and ventromedial hypothalamus (VMH) (Lukas et al. 2010). Overall, these studies illustrate the broad effects of early life manipulations achieved through long and brief maternal separations.

Similarly, variations in maternal behavior have been demonstrated to exert long-term influences on dopaminergic, GABAergic, glutamatergic, oxytocin and vasopressin neuropeptide systems, and brain-derived neurotrophic factor (BDNF). Offspring of Low LG dams have elevated stress-induced dopamine release within the mPFC (Zhang et al. 2005). Variations in maternal behavior in the rat and between different strains of mice have been shown to regulate GABAA receptor subunit composition with implications for its receptor pharmacology (Caldji et al. 2000, 2003, 2004). Offspring of Low LG dams exhibit a deficit in NMDA hippocampal subunit mRNA expression as adults (Bredy et al. 2003, 2004; Liu et al. 2000). Similarly, in biparental species, reduced paternal contact results in increased NR2A and decreased NR2B NMDA subunit mRNA expression in the hippocampus (Bredy et al. 2007). Male offspring of High LG rat dams have elevated levels of V1Ar in the amygdala (Francis et al. 2002b), whereas communally reared female mice have reduced V1Ar binding in the LS (Curley et al. 2009). Communally rearing and the experience of High LG are associated with elevations in hypothalamic OTR of female offspring (Champagne et al. 2001; Curley et al. 2009; Francis et al. 2000). Finally, hippocampal levels of BDNF have been demonstrated to decrease in response to maternal separation and complete maternal deprivation (Burton et al. 2007; Roceri et al. 2002), and increase in response to communal rearing in mice (Branchi et al. 2006a, b) and high LG in rats (Liu et al. 2000). Social modulation of these target systems has implications for response to novelty, anxiety-like and social behavior, and cognition, such that these early life experiences can achieve diverse developmental effects that persist into adulthood.

The translation of these laboratory-based findings to primate and human studies has provided further support for the impact of social experiences on brain region-specific activation, neuropeptide/neurotransmitter levels, and variations in gene expression. As it is the case for early deprivation paradigms in rodents, reductions in mother–infant contact in primates have a profound impact on the HPA response to stress. Heightened stress-induced cortisol and decreased rhythmicity in basal cortisol release are common features of rhesus monkeys reared in the absence of maternal stimulation, typically using a peer-rearing strategy (Barr et al. 2004; Suomi 1991). Compared with mother-reared rhesus monkeys, peer-reared infants showed increases in the volume of stress-sensitive brain regions, such as the dorsomedial prefrontal cortex and dorsal anterior cingulate cortex (ACC), in later

life (Spinelli et al. 2009). Peer-reared infants also have decreased serotonin transporter binding in the hypothalamus, caudate and putamen, globus pallidum, anterior cingulate gyrus, amygdala, and hippocampus (Ichise et al. 2006); decreased CSF 5-HIAA concentrations (Shannon et al. 2005); and an altered density of 5-HT1A receptors (Spinelli et al. 2010). Exposure of infant marmosets to repeated separations from parents is associated with long-term changes in gene expression, with particular effects on 5-HT1A mRNA levels within the ACC and hippocampus (Law et al. 2009a, b). Comparisons between nursery- and mother-reared infants indicate decreased CSF oxytocin levels in maternally deprived rhesus monkeys, which may account for the decreased social behavior observed in nursery-reared infants (Winslow 2005). Disruptions of mother–infant interactions, through use of a variable foraging demand, have been demonstrated to increase CSF levels of CRH (when exposure occurs in infancy; Coplan et al. 2001), induce elevations in CSF 5-HIAA and HVA (Coplan et al. 1998), and alter metabolism within the ACC (Mathew et al. 2003). Likewise, natural variation in maternal rejection rates is associated with altered serotonergic activity in offspring (Maestripieri et al. 2005, 2006). Thus, studies in primates have confirmed the neurobiological pathways that had previously been implicated in rodent models of abuse, neglect, and variation in parental behavior.

Advances in the development of noninvasive strategies for studying the impact of early life experiences on neural systems in humans have provided opportunities for translational studies on social modulation of the brain. Childhood neglect and abuse are associated with increased HPA activity and increased pituitary volume (Fries et al. 2008; Gerra et al. 2008; Neigh et al. 2009). CSF levels of 5-HIAA and HVA have been show to be negatively correlated with retrospective self-report scores of childhood emotional neglect (Roy 2002). Reduced levels of plasma BDNF associated with childhood neglect have also been reported in depressed patients and may account for cognitive impairments observed in these subjects (Grassi-Oliveira et al. 2008). Neuroimaging studies of Romanian adoptees that experienced severe neglect associated with institutionalization in infancy have indicated decreased overall white- and gray-matter volume and increased amygdala volume (Mehta et al. 2009). Positron emission tomography (PET) analysis indicates decreased metabolic activity within the orbital frontal gyrus, the infralimbic prefrontal cortex, amygdala, hippocampus, the lateral temporal cortex, and the brain stem of Romanian orphans (Chugani et al. 2001). Levels of vasopressin and oxytocin have also been found to be blunted in children who experienced early neglect (Fries et al. 2005). Variations in retrospective reports of parental care have also yielded significant negative associations with cerebrospinal levels of CRH (Lee et al. 2006). In nonclinical subjects, high levels of maternal care are associated with reduced trait anxiety and decreased salivary cortisol in response to stress, whereas low levels of maternal care are associated with increased DA release in the ventral striatum in response to stress (Pruessner et al. 2004). Other studies using the PBI have found a positive relationship between gray matter volume in the left dorsolateral prefrontal cortex (DLPFC) and paternal care score, whereas paternal and maternal overprotection were negatively correlated with the volume of this region (Narita et al. 2010). In a longitudinal study, observational ratings of parental

nurturance at age 4 predicted hippocampal volume in adolescence (Rao et al. 2010). These neurobiological studies of the effect of early life adversity suggest the persistent influence of these experiences on neural systems that regulate anxiety, social behavior, and cognition with implications for risk of later life psychiatric disorders.

### *10.1.3 Epigenetic Mechanisms and the Long-Term Effects of Social Experiences*

The biological pathways, though which early life social experiences exert such a profound neurobiological and behavioral impact, are being explored within many of the rodent, primate, and human experimental designs described in the previous sections. Although there may certainly be neuroanatomical alterations through which these effects are achieved, one approach to advancing our understanding of the association between the social environment and phenotypic variation comes from experimental designs incorporating the study of epigenetic regulation of gene activity. This epigenetic regulation of transcription is a critical feature of the link between genotype and phenotype and refers to those factors which control accessibility of DNA to transcription and which can alter the levels of gene expression (either silencing genes or increasing transcriptional activity) without altering the sequence of DNA. The molecular mechanisms through which these epigenetic effects are achieved include, but are not exclusive to, histone protein modifications and DNA methylation (Feng et al. 2007; Razin 1998). Within the cell nucleus, DNA is wrapped around a core of histone proteins, which can undergo multiple post-translational modifications including methylation, acetylation, and ubiquination (Peterson and Laniel 2004; Zhang and Reinberg 2001). These modifications alter the dynamic interactions between the histones and DNA, which either reduce or enhance the accessibility of DNA to transcription factors and RNA polymerase. In particular, histone acetylation is associated with increased transcriptional activity, whereas histone deacetylation or methylation is typically associated with transcriptional repression. Acetylation of histones is mediated by the enzyme histone acetyltransferase (HAT), whereas histone deacetylase (HDAC) promotes removal of the acetyl group from the histone tails. Thus, through alterations in the conformation of histones, the accessibility of DNA can be rapidly and reversibly altered. In contrast, DNA methylation has the potential to be a more stable and enduring modification to the activity of genes. DNA methylation occurs when cytosine nucleotides, usually located in CpG islands, are converted to 5-methylcytosine. This process is mediated by methyltransferases, which promote either maintenance (i.e., DNMT1) or de novo DNA methylation (i.e., DNMT3) (Feng et al. 2007; Razin 1998; Turner 2001). The conversion to 5-methylcytosine does not alter the DNA sequence, but can alter the likelihood that the gene will be transcribed and reduce transcription factor-mediated responses, particularly when methylation occurs

within gene promoter regions. Methylated DNA attracts methyl-binding proteins, such as MeCP2, which further reduce the accessibility of the gene and are associated with transcriptional repression (Fan and Hutnick 2005). The stability of DNA methylation patterns within the genome permits the stable regulation of gene expression associated with cellular differentiation and the heritability of this modification can be observed during mitotic cell divisions (Fukuda and Taga 2005).

Investigations of the role of epigenetic mechanisms in maintaining changes in gene expression induced by the early social environment have been explored in rodent experimental designs that model maternal abuse, maternal separation, and variations in mother–infant interactions during postnatal development (see Fig. 10.2). Daily exposure to abusive social interactions leads to reduced expression of BDNF in the prefrontal cortex in adulthood associated with increased DNA methylation within the BDNF IV promoter region (Roth et al. 2009). The functional importance of DNA methylation in mediating the long-term effects of abuse is further supported by findings that central administration of zebularine, a compound that reduces DNA methylation, leads to increased BDNF expression in maltreated rats such that BDNF levels are equivalent among abused and nonabused offspring. Daily and prolonged maternal separation has effects on a broad range of neurotransmitter and neuropeptide systems, and in a recent study, significant increases in AVP mRNA in the parvocellular neurons of the PVN was explored in maternally separated mice (Murgatroyd et al. 2009). Within the AVP gene, there are four regions rich in CpG islands that may regulate gene expression through DNA methylation. Analysis of PVN tissue indicated that at one of the four regions (CGI3), maternally separated males have significantly reduced DNA methylation

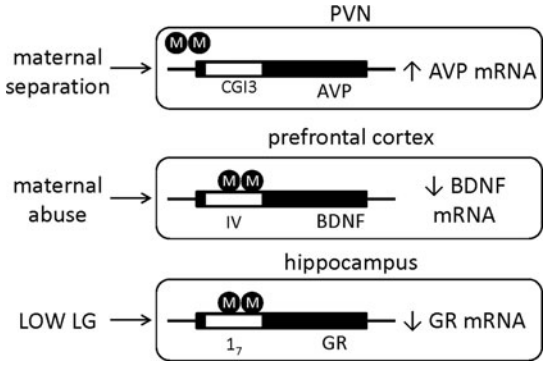


**Fig. 10.2** Epigenetic effects of postnatal environmental experiences. In rodents, postnatal maternal separation is associated with decreased DNA methylation within the AVP promoter region leading to increased AVP mRNA in the PVN (Murgatroyd et al. 2009), whereas maternal abuse is associated with increased methylation within the BDNF promoter in the prefrontal cortex leading to decreased BDNF expression (Roth et al. 2009) and the experience of low levels of LG in infancy is associated with increased methylation in the GR promoter region leading to decreased hippocampal GR expression (Weaver et al. 2004)

compared with control males at 6 weeks, 3 months, and 1 year of age. Furthermore, this hypomethylation was significantly correlated with increased mRNA expression, and these effects were brain region-specific as no changes in AVP mRNA or DNA methylation were found between maternally separated and control males in the supraoptic nucleus (SON). Analysis of the time course of the molecular changes involved in this differential methylation suggests that short-term activation of MeCP2 may be critical within the pathways leading to AVP hypomethylation and increased AVP mRNA levels within the PVN (Murgatroyd et al. 2009). Conversely, in response to brief maternal separation (handling), reductions in CRH mRNA in the parvocellular neurons of the PVN can be observed as early as PN9 (Korosi et al. 2010). Within the regulatory region of the CRH gene, there is a binding element for the repressor neuron-restrictive silencer factor (NRSF) (Seth and Majzoub 2001). This factor recruits cofactors and other enzymes/proteins involved in epigenetic regulation leading to the repression of gene expression (Zheng et al. 2009). Among handled offspring, protein levels of NRSF are dramatically higher in PVN tissue at PN9 and throughout adulthood, suggesting a possible mechanism for the initiation and maintenance of reduced CRH gene expression in response to handling-induced stimulation of mother–infant interactions.

Natural variations in postnatal maternal LG in the rat are associated with changes in numerous receptor pathways, with effects on hippocampal GR being implicated in the high levels of HPA reactivity observed among offspring of Low LG dams (Liu et al. 1997). Analysis of the GR $1_7$ promoter region suggests that low levels of LG are associated with increased GR $1_7$ methylation, decreased GR expression and an increased HPA response to stress. Time course analysis has indicated that these maternally induced epigenetic profiles emerge during the postnatal period and are sustained into adulthood (Weaver et al. 2004). The pathways through which these effects are achieved are currently being elucidated and it appears likely that maternal LG mediated up-regulation of nerve growth factor-inducible protein A (NGFI-A) in infancy may be critical to activating GR transcription and maintaining low levels of DNA methylation within the GR $1_7$ promoter among the offspring of High LG dams (Weaver et al. 2007). These experience-dependent shifts in epigenetic regulation of target genes within HPA stress pathways have also been observed to emerge prenatally in response to maternal exposure to chronic variable stress. Male offspring of mice exposed to gestational stress have decreased DNA methylation of the CRH gene promoter and increased methylation of the GR exon $1_7$ promoter region in hypothalamic tissue (Mueller and Bale 2008). These epigenetic modifications are associated with exposure to stress during the early stages of prenatal development and may involve dysregulation of placental gene expression. These findings complement studies in humans illustrating that methylation status of the GR promoter, particularly at the NGFI-A-binding site, in cord blood mononuclear cells of infants is associated with exposure to third trimester maternal depressed mood. Maternal depression was found to be associated with increased GR 1F promoter methylation in fetal blood samples and these methylation patterns predicted HPA reactivity in infants at 3 months of age (Oberlander et al. 2008). The susceptibility of HPA responses to social modulation

suggests that genes within these stress-sensitive pathways may be the target of epigenetic dysregulation associated with many forms of early life adversity.

### *10.1.4 Beyond Infancy: Epigenetic Effects of Juvenile and Adult Social Experiences*

Although social modulation of brain and behavior has been primarily explored in response to early social experiences, and in particular, mother–infant interactions, there continues to be plasticity in response to social experiences occurring during juvenile development and into adulthood. Social isolation during the postweaning period has generally been found to increase anxiety-like responses, though this may not involve the same neuroendocrine pathways targeted by earlier social experiences (Francis et al. 2002a; Lukkes et al. 2009). In rodents, postweaning social isolation has been associated with decreased expression of several 5-HT receptor subtypes in the prefrontal cortex, hypothalamus, and midbrain (Bibancos et al. 2007); latent elevations in DA levels in the NAc (Shao et al. 2009); reduced GABAA/CBZ receptor binding (Insel 1989; Miachon et al. 1990); decreased neuronal plasticity associated with glutamatergic hypofunction (Lu et al. 2003; Silva-Gomez et al. 2003; Stranahan et al. 2006); and sex-specific effects on the numbers of OT-positive neurons in the PVN (Grippo et al. 2007a, b). This cascade of neuroendocrine changes is associated with a phenotype referred to as an "isolation syndrome," which can be attenuated by treatment with antidepressants (Heritch et al. 1990). In contrast, juvenile environmental enrichment (typically involving both physical and social environmental complexity) in rodents attenuates the HPA response to stress with a concomitant decrease in basal corticosterone levels (Belz et al. 2003), and there is evidence for enrichment-induced elevations in hippocampal NGFI-A and GR (Olsson et al. 1994). In addition, social enrichment during juvenile development is associated with increased levels of the DAT within the NAc (Zakharova et al. 2009), increased GAD enzyme activity and extracellular GABA concentrations within the hippocampus (Frick et al. 2003; Segovia et al. 2006), increased AMPA receptor expression in the hippocampus (Bredy et al. 2003, 2004), and elevated OTR binding in a number of forebrain and hypothalamic areas including the central nucleus of the amygdala (Champagne and Meaney 2007). The social enrichment paradigm has been used to reverse deficits associated with pre- and postnatal environmental exposures (Francis et al. 2002a; Laviola et al. 2004; Morley-Fletcher et al. 2003) and augment phenotypes associated with targeted genetic manipulations (Jankowsky et al. 2005; van Dellen et al. 2000). Interestingly, recovery of memory deficits induced through p25-mediated neuronal loss can be achieved through exposure to complex housing environments and this enrichment is associated with increased histone (H3 and H4) acetylation in the hippocampus and cortex (Fischer et al. 2007). Moreover, treatment with histone deacetylase inhibitors can mimic the effects of environmental enrichment on learning and synaptic plasticity. These findings suggest the role of epigenetic mechanisms in shifting gene expression and behavior at these later stages of development.

In adulthood, chronic social defeat has been used to illustrate the continued influence of social experiences on neurobiological outcomes. This form of social stress, in which an individual has prolonged exposure to agonistic behavioral encounters, is associated with disruptions in social and emotional responding (Keeney and Hogg 1999). In adulthood, even a single social defeat is associated with prolonged alterations to the HPA stress response and changes to the expression of CRH receptors (Buwalda et al. 1999; Cooper and Huhman 2007). Social defeat results in transient changes in GABA receptor levels in cortex, cerebellum, and hypothalamus (Miller et al. 1987); increases in NMDA and decreases in AMPA receptor binding in the hippocampus (Krugers et al. 1993); and increases in expression of AVP mRNA in the PVN (Erhardt et al. 2009). Exploration of the epigenetic pathways linking the experience of social defeat to the behavioral phenotype that emerges in response to this adult social stressor has focused primarily on BDNF, which serves as a trophic factor that is a common downstream mediator of the effects of the multiple neurotransmitter and neuropeptide systems. BNDF gene expression is significantly decreased in the hippocampus of socially defeated male mice and this effect appears to be mediated by specific decreases in the BDNF III and IV transcripts (Tsankova et al. 2006). These effects are observed a month following exposure to the social stress, indicating a persistent effect on gene expression. Chromatin immunoprecipitation (ChIP) analysis indicates increased histone H3-K27 dimethylation at the BDNF III and IV promoters among socially defeated males, which may account for the reduced BDNF expression. Histone deacetylase (HDAC5) mRNA levels are also found to be decreased in socially defeated males (Tsankova et al. 2006) and HDAC5 appears to be important in mediating the effects of antidepressant treatment in males exposed to chronic social stress (Renthal et al. 2007). The differential levels of histone H3-K27 dimethylation are also found across the genome within the NAc, both in response to chronic social defeat and prolonged adult social isolation (Wilkinson et al. 2009). Analysis of histone acetylation in the NAc indicates that H3-K14 acetylation is initially decreased and then increased following chronic social defeat associated with decreases in HDAC2 levels. These studies suggest that though there is plasticity beyond the postnatal period, and that epigenetic mechanisms are responsive to juvenile and adult social experiences, dynamic histone modifications may be more evident in response to these later life experiences.

### *10.1.5 Transgenerational Epigenetic Effects*

Although use of the term "epigenetic" has its origin in the study of development and the notion that divergent gene activity plays a critical role in phenotypic variation, more recent conceptualizations of "epigenetic" are derived from the root "genetic" meaning the study of the units of heritable material (Jablonka and Lamb 2002). The notion that meiotic inheritance can be considered outside the realm of the DNA sequence is an area of growing philosophical and scientific interest, and there are

two distinct pathways via which epigenetic modifications are currently believed to be involved in the transmission of traits across generations. The first pathway, often referred to as epigenetic inheritance, involves incorporation of an epigenetic mark into the DNA which is then transmitted and perpetuated in subsequent generations through the germline (Morgan et al. 1999). The second pathway builds on the role of experience-dependent epigenetic modifications in developmental plasticity illustrated in the previous sections. Through these pathways, DNA methylation has been demonstrated to play a critical role in the transgenerational impact of early life experiences. The role of social experiences in shaping transgenerational effects is associated with experience-depended changes in the activity of genes that will, in adulthood, alter the reproductive behavior of females, leading to variations in the quantity and quality of mother–infant interactions (Champagne 2008). Natural variations in postnatal maternal care have been associated with altered gene expression and receptor levels within the MPOA, a brain region that is critical for maternal behavior (Fleming 1986). Females reared by Low LG dams have a reduced sensitivity to estrogen-mediated increases in neuronal activation within the MPOA (Champagne et al. 2001, 2003b) and analysis of levels of ERα in the offspring of High and Low LG dams suggest that differences in estrogen sensitivity are mediated by variations in ERα levels such that expression of ERα in the MPOA of both lactating and nonlactating female offspring of Low LG dams is significantly reduced (Champagne et al. 2003b). Analysis of levels of DNA methylation within the 1B promoter region of the ERα gene in MPOA tissue indicates that the experience of High LG is associated with decreased promoter methylation, whereas Low LG is associated with increased promoter methylation, leading to reduced gene expression and an attenuated response to hormonally primed behaviors (Champagne et al. 2006). ChIP assays demonstrate that this differential DNA methylation has consequences for the binding of transcription factors such as STAT5a to the 1B promoter. Maternal LG is associated with increased levels of STAT5a during the postnatal period and the increased levels of this factor may lead to sustained activation of transcription and reduced DNA methylation (Champagne et al. 2006). As a consequence of these epigenetic modifications, individual differences in maternal LG are transmitted from mother to offspring (F1 generation) and to grand-offspring (F2 generation) (Champagne and Meaney 2007; Champagne 2008). A similar experience-dependent transmission of behavior is observed in response to exposure to abuse. Female rat pups exposed to abusive caregiving in infancy engage in abusive caregiving toward their own offspring and F2 offspring of these F1 females have elevated levels of methylation within the BDNF promoter in the PFC and hippocampus (Roth et al. 2009). Interestingly, postnatal cross-fostering of F2 females to nonabusive dams did not reverse these epigenetic effects, suggesting that there may be prenatal factors that contribute to the generational transmission of altered DNA methylation patterns. The transgenerational inheritance of stable individual differences in behavior, mediated through epigenetic mechanisms, provides an alternative route of inheritance of phenotype that may allow for the environmental conditions and social experiences of previous generations to influence development.

### *10.1.6 Concluding Remarks*

Development occurs within a social context and there is increasing evidence that epigenetic mechanisms may play a critical role in linking experiences to long-term neurobiological changes. Although much of the evidence supporting the hypotheses that DNA methylation and histone modifications are altered by the social environment has come from studies in rodents, translational studies are emerging, which suggest that, for example, the experience of abuse in infancy can lead to epigenetic variation in the human brain (McGowan et al. 2008, 2009). Thus, the animal models of abuse, neglect, and variation in parental care that have been used to study social modulation of the developing brain can continue to inform and inspire hypothesis-driven epigenetic research in humans. Given the brain region specificity of many of the gene expression changes that have been observed in these models, one critical question that must be addressed relates to the ability to predict epigenetic changes in the brain using peripheral markers of transcriptional activity measured in blood lymphocytes. Establishing this relationship will enable the application of an epigenetic approach to longitudinal studies in humans where in vivo changes in DNA methylation and histone modifications can be associated with variations in social experience. In addition, the plasticity of the epigenome in response to both behavioral and pharmacological intervention in later life that has been observed in rodent studies (Roth et al. 2009; Weaver et al. 2004, 2005) may provide a novel therapeutic approach to the treatment of disorders related to early life adversity. The dynamic yet stable nature of epigenetic variation may be a critical feature of both within- and across-generation individual differences in phenotype that expand our concept of the origins of variation in brain and behavior.

**Acknowledgment** This work was supported by Grant Number DP2OD001674 from the Office of the Director, National Institutes of Health.

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# Chapter 11
# Toward an Understanding of the Dynamic Interdependence of Genes and Environment in the Regulation of Phenotype

## Nurturing our Epigenetic Nature

**Ian C.G. Weaver**

**Abstract** Developmental plasticity refers to the potential for intraindividual change. Traditionally, the relationship between the human genome and the environment has been presented under the framework of gene–environment interactions. However, adaptive phenotypic plasticity emerges from more than just genotype. In humans and nonhuman primates, the nature of mother–infant interactions early in life has a profound role in mediating variation in offspring phenotype, including emotional and cognitive development, which is endured through life. One critical question: How is this "environmental programming" established and maintained in the offspring? Evidence from rodent studies suggests that maternal care in the first week of postnatal life establishes diverse and stable phenotypes in the offspring through epigenetic modification of genes expressed in the brain, which shape neuroendocrine and behavioral stress responsivity throughout life. This research demonstrates that the epigenetic state of a gene can be established through early in life experience and is potentially reversible in adulthood. These findings may well form the molecular basis for understanding potential mechanisms of environmental and developmental determinates of individual differences in human stress reactivity and health outcomes. Henceforth, epigenetic modifications of specific genomic regions in response to variations in environmental conditions might serve as a major source of variation in biological and behavioral phenotypes.

**Keywords** Chromatin · DNA methylation · Glucocorticoid receptor · Hippocampus · Maternal care · Stress · Transgenerational inheritance

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_11,
© Springer-Verlag Berlin Heidelberg 2011

## 11.1 Early Life Development: Making an Intraindividual Difference

The study of development is an examination of adaptation and therein the biological basis of variation among alternative phenotypes. Phenotype is maintained through multifarious interactions between the dynamics of cellular-level function in response to intrinsic and extrinsic (environmental) cues. The cells of an organism contain the same genotype but are structurally, functionally, and phenotypically heterogeneous in response to different spatial and temporal regulation of gene expression profiles influencing multiple cellular functions including differentiation and morphogenesis (Baccarelli and Bollati 2009; Dempfle et al. 2008; Ishibe and Kelsey 1997; Kraft and Hunter 2005; London and Romieu 2009). The generation of different degrees of individual adaptive modification from a single genome in response to changes in the environment – either stochastic or predictable – forms the basis for "phenotypic plasticity", which is apparent across all froms of life.

In altricial species such as humans, the transition from embryo to adulthood is a lengthy process, and its evolutionary development has been long and complex (Saccheri and Hanski 2006). Embryonic development manifests as a series of changes in cellular programs that mark the transitions from the totipotent zygote to increasingly more differentiated cells with specialized morphology and function. As an individual progresses through prenatal (embryonic–fetal combined), neonatal, infancy, childhood, juvenile, puberty, adolescence, prime, and senescence – from dependency to increasing autonomy – physiological and neurodevelopmental systems continually receive, transform, and update information regarding the demands of the environment (Oli and Dobson 2003; Roff 2002). The physiological homeostatic set points of the internal environment of an individual are controlled by multiple systems (cytokines, growth factors, hormone secretion, and neurotransmitter release), and these interactions apply to the brain.

The stability of a child's early life conditions both before birth and in infancy has profound effects on their long-term physical and mental health outcomes. The relationship between the quality of early environment and long-term developmental programming appears to be mediated, in part, by the closeness or degree of positive attachment in parent–infant bonding and parental investment during early life (Canetti et al. 1997). Critical phases in early life are characterized by dynamic responses to chemical, biological, and physical stimuli (i.e., nutritional restriction, gestational diabetes, and maternal stress) that permanently alter (or "program") gene expression profiles contributing to the organization and function of neural circuits and molecular pathways supporting biological mechanisms (intrauterine growth, physiology, and metabolism) and psychological processes (socialization and intellectual maturation) in the offspring (Barker et al. 2002; Fowden et al. 2006; Huizink et al. 2004; Mousseau and Fox 1998). The ability of the early environment to modify adult phenotypes (neuroendocrine, behavioral, emotional, metabolic, and cognitive) via alterations in mother–infant interactions allows the transmission of information involved in

enhanced survival to be passed on to offspring without having to go through the slow processes of random mutation and natural selection. This implies that plasticity early in life provides the individual with an evolutionary advantageous ability to adjust physiologically and hone specific biological defensive systems for survival and reproductive success to promote establishment and persistence in the present environment (Bradshaw 1965).

Depending on how close the plastic response is to the new favored phenotypic optimum dictates whether directional selection will cause adaptive divergence between populations (Robinson et al. 2008). Plasticity can become maladaptive under conditions where environmental stimuli programs physiological systems to function outside their normal range, which increases the biological systems "wear and tear" (or allostatic load) and risk for diseases that occur with greater frequency with aging (McEwen 2004). Herein, developmental programming mediates the effects of experience on vulnerability (and resilience) to the emergence of certain metabolic disorder phenotypes in the later stages of life (Coe and Lubach 2005; Gluckman et al. 2008). This begs the question of how the individual is capable of adapting to these developmental or environmental cues at the level of the genome, including the biological mechanism(s) through which these responses are established and maintained.

Genetic variation for phenotypic plasticity has been demonstrated in a number of different species, suggesting that it is open to the forces of natural selection, and that adaptive phenotypic plasticity can evolve (West-Eberhard 2005). However, phenotypic plasticity is more than the simple property of genotype. Indeed, there are multiple potential mechanisms of inheritance, involving the passage of epigenetic marks through the germ line (Chong and Whitelaw 2004); the passage of maternal RNA molecules into the embryo (Bettegowda and Smith 2007; Rassoulzadegan et al. 2006); the potential passage of prion proteins from parent to offspring (Shorter and Lindquist 2005); the biochemical state of the gametes at the time of conception; and the transmission of nutrients, bacteria, or antibodies from maternal circulation to that of the offspring (Boulinier and Staszewski 2008; Grindstaff et al. 2003; Hasselquist and Nilsson 2009). All of these factors can, and do, influence the phenotype of the offspring.

Epigenetics investigates the transmission of phenotypic characteristics in terms of gene expression through mitosis (and potentially meiosis) in the absence of changes in DNA sequence, hence the name epi- (Greek: επί – over, above) genetics (Waddington 1942). The advent of high-throughput techniques such as microarray- and sequencing-based approaches to study the distributions of regulators of gene transcription throughout the genome led to the collective description of the "epigenome", which refers to the epigenetically modified genome. The "epigenotype" refers to mitotically heritable patterns of DNA methylation and modifications to chromatin proteins that package DNA. Henceforth, phenotype is thought to be the result of complex interactions between genotype and current, past, and ancestral environment leading to life-long experience-dependent chromatin plasticity.

This chapter provides an overview of the main components of the epigenome and, using a rodent model of early life social interaction, discusses how epigenetic

programming through maternal care subsequently shapes brain development and behavioral function across the life span. Herein, socially directed regulatory processes transfer epigenetic information not only within cells but also between cells and organ systems, as well as across generations. In humans, the epigenome may well function as an interface between the inherited genome and the dynamics imposed by the environment, providing a mechanism for reprogramming gene function in response to changes in lifestyle trajectories, fundamental for adaptive phenotypic plasticity.

## 11.2 The Dynamic Epigenome: Linking Environment to Genes and Plasticity

Epigenetic events in eukaryotic organisms (plants, insects, reptiles, birds, and mammals) have evolved to provide a more precise and stable control of gene expression and genomic regulation through multiple generations. In eukaryotic cells, the epigenome is encoded in distinct patterns of nuclear organization, global chromatin structure, global and local covalent modification of both histone proteins (regular and variants) (Kadonaga 1998) and DNA (Razin 1998), and the presence of specific macromolecules including small nonprotein-coding RNAs (ncRNAs) termed microRNA [other ncRNA include Piwi-interacting RNAs and large intervening non-coding RNAs] (Bergmann and Lane 2003; Chuang and Jones 2007; Saito and Jones 2006). MicroRNA regulates gene expression and cellular fate by controlling chromatin silencing, mRNA stability, or translation, and potentially plays an important role in developmental neuropathology (for review see Mattick et al. 2009). For the purpose of this review, we discuss only DNA and histone modification, considering microRNA are regulated by DNA methylation and chromatin structure (Saito and Jones 2006). Unlike the DNA sequence, which is stable and strongly conserved, epigenetic processes can be highly dynamic: chromatin structure and DNA methylation patterns are unique to each type of cell, developmentally regulated, and often induced by exposure to a range of external environmental factors (Dolinoy et al. 2007). Understanding the mechanisms involved in the initiation, maintenance, and heritability of epigenetic states is an important aspect of research in current biology, particularly in the study of phenotypic behavior in humans.

### *11.2.1 Chromatin Structure, Modifying Enzymes, Histone Code, and Genome Function*

In the eukaryotic cell nucleus, chromosomal DNA is packaged into chromatin fibers in repeating protein–DNA complexes called nucleosomes, the basic unit of DNA packaging. Nucleosomes comprise approximately 146 bp of DNA wound 1.8 times around an octamer consisting of two copies each of histone proteins H2A, H2B, H3,

and H4 (Kornberg 1974). The attraction between the positively-charged histones and negatively-charged DNA maintains the histone–DNA interaction (Grunstein 1997).

The posttranslational modification of histones, the basic proteins around which DNA is wrapped to form nucleosomes, comprises an epigenetic mechanism related to gene expression. Histone-modifying enzymes are generally not gene specific. Transcription factors or repressors locate and bind to their cognate response elements on the genome (Elf et al. 2007) and concurrently recruit (via protein–protein interactions) specific chromodomain proteins and histone-modifying enzymes to these specific genomic regions. Notably, intracellular signal transduction pathways activated by cell-surface receptors can directly interact with the chromatin structure, linking environmental cues from cell-surface receptors to gene-specific histone posttranslational modification and the level of expression from the underlying genes.

Within the chromatin structure, posttranslational modifications not only occur on the protruding N-terminal tail of histones, but can also affect the histone core and C-terminus [e.g., H3 methylation of lysine 79 residues (H3K79me)] (Tweedie-Cullen et al. 2009). These modifications include acetylation (Wade et al. 1997), poly-ADP-ribosylation, carbonylation (Wondrak et al. 2000), citrullination, biotinylation, formylation, palmitoylation, glycosylation, methylation (Jenuwein 2001), phosphorylation, SUMOylation (Shiio and Eisenman 2003), proline isomerization, and ubiquitination (Shilatifard 2006). The addition or removal of these histone modifications are often regulated together (either positively or negatively), and act in synergy to modulate gene transcription (activation or repression) by altering local chromatin structure and access for transcriptional machinery. Typically, acetylation occurs at lysine residues and all acetylation modifications are associated with transcriptional activation, as are phosphorylation and arginine methylation modifications (Bernstein et al. 2005). The effect of ubiquitination on transcription likewise is dependent on location, with ubiquitination on H2A and H2B associated with transcriptional repression and activation, respectively. In unicellular eukaryotes such as the yeast *Saccharomyces cerevisiae*, histone sumoylation is also associated with transcriptional silencing (Nathan et al. 2006), and H3 proline isomerization with transcriptional activation via negative coupling with H3 methylation of lysine 36 residues (H3K36me) (Nelson et al. 2006). The relationship between regional patterns of histone modifications and locus-specific transcriptional activity provides evidence for the existence of a "histone code" for determining cell-specific gene expression programs (Jenuwein and Allis 2001).

Other chromatin remodeling systems that have been implicated in epigenetic changes include DNA looping, nucleosome sliding (mediated by ATP-dependent chromatin remodeling proteins), and histone substitution (exchange of histones from nucleosome with external histones) (Tsankova et al. 2007). For example, different histone variants, which replace the standard isoforms also play a regulatory role and, in some cases, serve to mark gene activation (Henikoff et al. 2004). The studies discussed in this chapter focus on the functions of H3 and H4 lysine acetylation and methylation (H3/4-KAc/me) with some general considerations concerning gene regulation, activation, and repression during development.

The occurrence of acetylation on histone tails is controlled by the opposing enzymatic activities of histone acetyltransferases [HATs; i.e., CREB-binding protein

(CBP)] and histone deacetylases (HDACs; i.e., the class I HDAC2) (Kuo and Allis 1998). Increased acetylation induces transcription activation – H3 acetylation of lysine 9 residues (H3K9Ac) at the 5′ region of genes is associated with promoter activation – whereas deacetylation usually induces transcription repression. Histone acetylation neutralizes the positive charge of the histone tail and decreases its affinity to negatively charged DNA, generating a more open DNA conformation (euchromatin) (Hong et al. 1993; Sealy and Chalkley 1978). This enables access of transcription factors, regulatory complexes, and RNA polymerase transcription factors to the DNA, and the expression of the corresponding genes is also facilitated. Thus, H3K9Ac is considered as a predominant marker of active gene transcription (Lee et al. 1993; Perry and Chalkley 1982). On the other hand, removal of the acetyl group by HDAC enzymes restores the positive charge to the lysine residue, fostering stronger interactions between histones and DNA (heterochromatin), reducing the accessibility of transcription factors, and the transcription apparatus to their cognate binding sites, resulting in gene silencing (Davie and Chadee 1998).

The amount of methylation on histone tails is controlled by the opposing enzymatic activities of histone methyltransferases (HTMs; i.e., EZH2, G9a, MLL, Suv39H1) (Lachner and Jenuwein 2002; Lachner et al. 2001; Lachner et al. 2003) and histone demethylases (HDMs; i.e., JARID1d, Utx) (Shi et al. 2004; Tsukada et al. 2006). Lysine residues can be mono- (me1), di- (me2), or tri-methylated (me3), and binding of specific proteins that recognize methylated lysine positions can result in different biological outcomes – some specific histone methylation events are associated with gene silencing and some with gene activation depending on the lysine residue (Yan and Boyd 2006). For example, H3 di- or tri-methylation of lysine 4 residues (H3K4me2/3) at the 5′ region of genes is associated with promoter activation, whereas H3 di- or tri-methylation of lysine 9 residues (H3K9me2/3) is associated with Dnmt3a activity, DNA methylation, and repressed gene transcription (Ohm and Baylin 2007). H3 tri-methylation of lysine 27 residues also (H3K27me3), which is catalyzed by the polycomb group (PcG) protein complex, represents transcriptional inactivation. Permissive and repressed intermediate chromatin states provide an additional level of epigenetic regulation (Tsankova et al. 2007).

### *11.2.2 Genomic Methylation: The Primary Epigenetic Modification*

Of DNA methylation, cytosine methylation is the best understood and the most stable epigenetic modification modulating the transcriptional plasticity of mammalian genomes. Recent studies examining whole-genome methylation profiles across the plant and animal kingdoms have revealed both conserved and divergent features of DNA methylation in unicellular eukaryotes to multicellular vertebrates (Feng et al. 2010b; Law and Jacobsen 2010; Zemach et al. 2010). In plants and mammals, the most methylated cytosines are found over repeat elements (Goll and Bestor 2005; Law and Jacobsen 2010; Suzuki and Bird 2008) and loss of this modification

is associated with transcriptional reactivation as well as increased mobilization of transposable elements (Slotkin and Martienssen 2007). These observations likely reflect the ancestral role of cytosine methylation in the defense against invasive DNA. Nonrepeat sequences may also be methylated, and methylation of such sequences in the context of gene promoters often correlates with transcriptional silencing in plants (Henderson and Jacobsen 2007) and mammals (Ooi et al. 2009; Suzuki and Bird 2008). Despite similarities in the controlling functions of DNA methylation, the dynamics and deposition of methylation patterns differ in several respects between plants and mammals.

In plant genomes, DNA methylation can occur symmetrically (both strands) at cytosine residues in both 5′-cytosine-phosphodiester-guanine-3′ (CpG dinucleotide) and CHG [H = adenine (A), thymine (T) or cytosine (C)] contexts, and also asymmetrically in CHH context, with the latter directed and maintained by RNAs (Law and Jacobsen 2010). Interestingly, in plants, methylation within genes (intragenically) is thought to inhibit cryptic transcription initiation (Zilberman et al. 2007) or suppress recombination or transposon insertion within genes (Zhu 2008).

In mammalian genomes, DNA methylation occurs mostly symmetrically at cytosine residues in the context of CpG dinucleotides in the mammalian genome, to form 5-methylcytosine (5mC) in a cell-specific pattern (Razin and Szyf 1984). However, results from more recent studies suggest that CHH and CHG methylation may be more common than previously appreciated in mammals: in human embryonic stem (ES) cells it accounts for ~25% of all methylated cytosines (Ramsahoye et al. 2000; Lister et al. 2009). In mammals, 5mC patterns are established and maintained during development by the Dnmt1 and Dnmt3 families of DNA methyltransferases, which utilize the final methyl donor produced by one-carbon metabolism, *S*-adenosylmethionine (also known as SAM, SAMe, ademethione, and adoMet) (Adams et al. 1979). The de novo establishment of DNA methylation is performed by methyltransferases Dnmt3A and Dnmt3B, and modulated by Dnmt3L, which lacks direct catalytic activity (Okano et al. 1999). These enzymes are all expressed in the central nervous system (CNS) and are dynamically regulated during development and differentiation (Feng et al. 2005; Goto et al. 1994). Indeed, Dnmt1 actively methylates CpG dinucleotides within nondividing somatic cells, such as neurons (Okano et al. 1999).

Importantly, global levels of 5mC and gene-specific DNA methylation profiles are dynamic and vary spatially and temporally throughout life, especially during epigenetic remodeling in early development. The Dnmt1 maintenance methyltransferase shows preference hemi-methylated DNA (Bestor and Verdine 1994; Leonhardt and Bestor 1993; Smith 1994) and safeguards the methylome in dividing cells by faithfully copying the methylation pattern from parental to daughter strand during DNA replication (Bestor 1988; Bestor 1992). This process is especially important during embryogenesis, where the methylated maternal and paternal genomes are demethylated upon fertilization (to ensure the totipotency) and specific patterns of methylation are then reestablished progressively commencing in the early postconception period (Okano et al. 1999). Recent work in human cell lines has also shown that dynamic remodeling of epigenetic marks may occur during the cell cycle (Brown and Szyf 2008; Kangaspeska et al. 2008; Metivier et al. 2008).

As previously mentioned, the addition of a methyl group to cytosine nucleotides in DNA does not change the primary DNA sequence per se, but the covalent modification can regulate spatio-temporal gene expression and activity in a mitotically heritable fashion. Through this mechanism, DNA methylation controls cell-specific gene expression, X chromosome inactivation and parental imprinting, cell survival, neuronal migration and maturation in the CNS, and can be modulated by environmental stimuli such as nutrition, drugs, stress, and postnatal care (for review see Weaver 2009). Furthermore, epigenetic regulation through DNA methylation appears to be a critical evolutionary process – the absence of DNA methylation in some eukaryotes such as yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, as well as the flatworm *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster* is associated with the evolutionary loss of DNMT homologues (Goll and Bestor 2005).

### *11.2.3 Heterochromatin, DNA Methylation, and Gene Silencing*

Typically, heterochromatin is associated with hypermethylated DNA and inhibition of transcription (Holliday and Pugh 1975). During early development, for example, repressive heterochromatin is important for X chromosome inactivation (or lyonization) during embryonic development to ensure that females, like males, have one functional copy of the X chromosome in each cell (Orphanides and Reinberg 2002).

DNA methylation of gene promoter regions or enhancer sites inhibits gene expression via two main mechanisms (Bird 2001; Bird and Wolffe 1999; Hashimshony et al. 2003; Kadonaga 1998; Li 2002; Nan et al. 1998). In the direct mechanism, the 5mC marking of CpG-rich promoters (Antequera and Bird 1993; Bird 1996; Gardiner-Garden and Frommer 1987), intragenic, and intergenic regions (Ching et al. 2005; Fazzari and Greally 2004; Khulan et al. 2006) blocks gene expression, thereby establishing a mechanism to direct tissue-specific gene expression – 5mC within transcription factor binding sites displaces the binding of methylation-sensitive transcription factors to their cognate binding sites (Tate and Bird 1993; Watt and Molloy 1988). Here, DNA methylation serves as an epimutation of the transcription factor binding site and repels the transcription factor. DNA methylation within intronic regions may regulate the activity of intragenic microRNA involved in regulating RNA splice variation, silencing of chromatin, degradation of mRNA, and blocking translation (Mattick and Makunin 2006). In the indirect mechanism, the methylation sites attract methyl-CpG-binding domain (MBD) containing proteins, such as methyl-CpG-binding protein (MeCP)-2, which are involved in "reading" methylation marks and binding to methylated DNA (Bird 2001; Bird and Wolffe 1999; Hashimshony et al. 2003; Kadonaga 1998; Li 2002; Nan et al. 1998). These MBD proteins affect chromatin condensation by recruiting corepressor proteins such as SIN3A and histone modification enzymes, leading to chromatin compaction and gene silencing (Jones et al. 1998; Ng et al. 1999; Wade et al. 1999; Zhang et al. 1999).

### *11.2.4 Euchromatin, Active DNA Demethylation, and Gene Activation*

Similar to histone modification, DNA methylation is also potentially reversible during development and in somatic tissues (Kersh et al. 2006; Lucarelli et al. 2001) including predominantly postmitotic tissues such as the brain (Feng et al. 2010a; Weaver et al. 2004). Hypomethylated DNA is generally associated with euchromatin and gene activation (Holliday and Pugh 1975).

As discussed previously, in the absence of maintenance and de novo DNA methylation activity, DNA methylation patterns are lost passively during DNA replication in primordial germ cells (Morgan et al. 2005). However, the precise mechanism by which replication-independent (or active) demethylation occurs in the adult mammalian brain remains as a subject of a lively ongoing debate (Ooi and Bestor 2008; Wu and Zhang 2010). One proposed mechanism involves nucleotide excision repair – the removal and replacement of mutations in the DNA and the growth arrest and DNA damage-inducible 45 (Gadd45a) protein has been proposed to promote the DNA repair based mechanism (Barreto et al. 2007; Weiss et al. 1996). In contrast, base excision repair involves the removal of a mutated or chemically altered base and its replacement with the correct base (David and Williams 1998). However, these two mechanisms may not account for the rapid and complete demethylation observed in the paternal genome following fertilization of the embryo (Oswald et al. 2000). The removal of nucleotides would seriously compromise the integrity of the genome, and it is unlikely an excision repair system that would be able to complete genome-wide demethylation and nucleotide replacement within the observed 4-h time frame (Oswald et al. 2000).

Interestingly, some components of the epigenetic machinery long thought to be involved in establishing and maintaining DNA methylation patterns may also be involved in their removal. This should not be unexpected as enzymes often have the potential for bidirectional catalytic activity. A recent publication provides evidence for DNA demethylation induced by MBD3 (Brown et al. 2008), while two other reports propose that DNMT3a and DNMT3b possess deaminase activity and are involved in a dynamic demethylation–methylation pathway that operates during gene transcription (Kangaspeska et al. 2008; Metivier et al. 2008). Prior to these assertions, MBD2b (a shorter isoform of MBD2) was reported to trigger active DNA demethylation by removal of methyl groups directly from the cytosine base and induce gene expression in mammalian cells (Bhattacharya et al. 1999; Cervoni et al. 1999; Cervoni and Szyf 2001; Detich et al. 2002, 2003a, b; Hamm et al. 2008; Ramchandani et al. 1999; Szyf and Bhattacharya 2002a, b). The proposed reaction requires a water molecule and involves the transfer of the methyl group of the cytosine to form methanol (Bhattacharya et al. 1999; Cervoni et al. 1999; Ramchandani et al. 1999). Although the assignment of demethylase activity to MBD2b was contested (Boeke et al. 2000; Ng et al. 1999; Wade et al. 1999), MBD2b levels are inversely correlated with the levels of DNA methylation of certain genes in hepatocytes (Goel et al. 2003) and lymphocytes from lupus patients (Balada et al. 2007), and depletion of MBD2b

results in hypermethylation of unmethylated genes in metastatic cancer (Pakneshan et al. 2004; Shukeir et al. 2006). Hence, the ability to epigenetically reprogram differentiated cells is becoming of major medical importance.

More recently, it has been shown that while 5mC constitutes only ~1% of all bases in the mammalian genome, the modified base 5-hydroxymethylcytosine (5hmC) constitutes ~5% of all cytosine species present at CpGs in MspI and TaqαI sites in ES cell DNA and 20% of all cytosine species present at CpGs in the brain, especially in Purkinje neurons (Kriaucionis and Heintz 2009; Tahiliani et al. 2009). Since ES cells are highly proliferative while neurons are postmitotic, the biological functions of 5hmC maybe cell type-specific and may influence chromatin structure and recruit specific factors or may constitute an intermediate component in cytosine demethylation. Herein, 5hmC may (1) displace 5mC-binding proteins (e.g., MeCP2) from methylated DNA (Valinluck et al. 2004); (2) occlude DNMT1 during cell division resulting in passive DNA demethylation (Valinluck and Sowers 2007); (3) be recognized as an aberrant base by DNA repair mechanisms that replace 5hmC with cytosine resulting in active DNA demethylation; or (4) be recognized by transcription factors that recruit and target chromatin-modifying enzymes to specific genomic regions, thus altering chromatin structure and DNA methylation status.

DNMT1-mediated oxidation of cytosine with formaldehyde may generate 5hmC, although this remains to be demonstrated under physiological relevant conditions (Liutkeviciute et al. 2009). Tahiliani et al. (2009) recently reported that the human mixed-lineage leukemia (MLL) fusion protein TET1 – which is an α-ketoglutarate (α-KG) and Fe(II)-dependent dioxygenase – specifically binds 5mC and catalyses the conversion to 5-hydroxymethylcytosine (5hmC) (Tahiliani et al. 2009). Ito et al. (2010) extend these studies by demonstrating that all three murine Tet proteins (Tet1–3) catalyze a similar reaction (Ito et al. 2010). Furthermore, Tet1 was shown to mediate the regulation of Nanog, which is a transcription factor critically involved with self-renewal of undifferentiated ES cells. Ectopic Tet1 expression resulted in increased Nanog promoter 5hmC and increased Nanog expression. Whether 5hmC is further processed to C by an enzyme-catalyzed process or through base excision remains to be determined. Notably, a 5hmC-specific DNA glycosylase activity has been previously reported (Cannon et al. 1988). Alternatively, decreased Tet1 expression in preimplantation embryos was associated with increased methylation of the Nanog promoter, decreased Nanog gene expression, and a bias toward trophectoderm differentiation (Ito et al. 2010). Together, these findings demonstrated a role for Tet1-mediated DNA demethylation during maintenance of pluripotent ES cell self-renewal and specification, including neural differentiation.

In summary, DNA methylation patterns are likely defined by chromatin status, which gates the accessibility of the DNA methylation/demethylation components to the underlying genes. This process may involve transcription factors or repressors that recruit and target chromatin-modifying enzymes to specific genomic regions. The chemical nature of the chromatin modification then defines the methylation status of the underlying genes, either through the facilitation of the DNA

demethylation or the recruitment of the DNA methylation machinery (Weaver et al. unpublished). Clearly the exact timing and mechanisms through which a given pattern of chromatin changes from transient effects on gene regulation to more persistent epigenetic programming of gene expression by DNA methylation will be context-dependent (Madhani et al. 2008).

### *11.2.5 Dsyregulation of the Epigenetic Machinery and Neurodevelopment*

Importantly, from a plasticity perspective, loss-of-function mutations in key components of the epigenetic machinery in humans are associated with several developmental and cognitive disorders with implications for risk of later life mental disorders (Jiang et al. 2004). Genetic variants of genes encoding the DNMT enzymes have been identified as risk factors of disease, including cancer (Cebrian et al. 2006; Kelemen et al. 2008; Lee et al. 2005; Montgomery et al. 2004), systemic lupus erythematosus (Park et al. 2004), and a rare autosomal recessive disorder termed ICF (immunodeficiency, centromeric instability, and facial anomalies) syndrome (Hansen et al. 1999), diseases which are characterized by altered DNA methylation patterns. In addition, functional polymorphisms of genes involved in folate metabolism [such as methylenetetrahydrofolate reductase (MTHFR), a regulatory enzyme in folate metabolism] have been shown to alter intracellular SAM levels (Miller et al. 1994; Poirier et al. 2001), decrease global DNA methylation levels in peripheral blood leukocytes (Friso et al. 2005), and to be linked to the increased risk of many serious health conditions (Giovannucci 2004). Thus, genotype is likely to be a further major influence on epigenotype.

The importance of epigenetic mechanisms in neurodevelopmental conditions including mental retardation has been the best characterized by mutations in the X chromosome-linked gene MeCP2 in individuals with Rett syndrome (RTT) (Amir et al. 1999), and mutations in the CBP gene (which has HAT activity) in individuals with Rubinstein–Taybi syndrome (RTS) (Alarcon et al. 2004).

Both MeCP2 and CBP are highly expressed in postmitotic neurons and are involved in regulating neural gene expression (Chen et al. 2003; Martinowich et al. 2003).

Mice with truncated MeCP2 exhibit genome-wide H3 hyperacetylation (H3Ac), neuronal atrophy, increased anxiety, cognitive deficits, and social withdrawal, which can be further exacerbated by forebrain knockout of the brain-derived neurotrophic factor (BDNF) (Shahbazian et al. 2002). Remarkably, many of the physiological, cognitive, and emotional deficits associated with MeCP2 mutant mice are reversed through restoration of the MECP2 gene (Guy et al. 2007), or by ectopic BDNF expression, demonstrating a functional interaction between MeCP2 and BDNF in RTT disease progression (Chang et al. 2006).

Mutations of the CBP HAT domain in several RTS cases are associated with genome-wide histone hypoacetylation and cognitive dysfunction in adulthood (Kalkhoven et al. 2003). The learning and memory deficits are attributed to

perturbed neural plasticity (Korzus et al. 2004), however, RTS individuals also exhibit early cognitive dysfunction (Roelfsema and Peters 2007) and display neural dysgenesis, including cortical abnormalities (Sener 1995). Similar to RTS in humans, mice with a heterozygous null mutation of CBP perform poorly in cognitive tasks and show decreased genome-wide histone acetylation (for review see Josselyn 2005). We examined the potential role for CBP in neural precursors born in the subventricular zone of the lateral ventricles of the developing murine cortex, which sequentially generate neurons, astrocytes, and oligodendrocytes (Wang et al. 2010). Herein, we found that phosphorylation of CBP by atypical protein kinase C (aPKC) ζ acts as an epigenetic switch to promote precursor differentiation. Interestingly, this epigenetic mechanism is perturbed in the fetal brains of CBP haploinsufficient mice, which exhibit early behavioral deficits as pups in ultrasound vocalization following maternal separation (Wang et al. 2010). These findings provide a novel mechanism whereby environmental cues, acting through histone modifying enzymes, can regulate stem cell epigenetic status and thereby directly promote differentiation, which regulates neurobehavioral development. This begs the question of whether similar epigenetic mechanisms regulate differentiation in other brain regions, such as in the small number of new neurons that arise from the subgranulare zone of the dentate gyrus within the hippocampus throughout life (Cameron and Gould 1994).

## 11.3 Experience-Dependent Effects on the Epigenome and Phenotypic Variability in Nature

There are a growing number of examples illustrating the relationship between epigenetic changes and phenotypic variability in divergent eukaryotic species (for review see Weaver 2010). The potential flexibility of the epigenome is a means of responding to changing environmental stimuli, and is especially important for plants, which cannot move in response to unfavorable conditions (Martienssen and Colot 2001). For example, in the flowering plant *Arabidopsis*, the cold-induced acceleration of flowering in different climates (vernalization) is correlated with the rate of epigenetic silencing by H3 tri-methylation of lysine 27 residues (H3K27me3) at the Flowering Locus C (FLC) gene (Shindo et al. 2006).

Another mode of epigenetic inheritance in plants is by paramutation: an epigenetic modification induced by cross-talk between allelic loci (Chandler 2007). The paramutation is inherited by transmitting the RNA transcript from the "paramutating" allele during gametogenesis and by propagating this heritable message to somatic and germ cells of the offspring by an RNA-dependent RNA polymerase (Alleman et al. 2006). Unlike a typical mutation, paramutation results in quantitatively variable phenotypes. Paramutation-like effects suggest the possibility of non-Mendelian heredity comparable to the plant systems, for example, the interallelic transfer of DNA methylation patterns established after heterologous recombination during meiosis (Rassoulzadegan et al. 2002). Currently, the only example of a paramutation

mode of epigenetic inheritance in mammals is in transgenic mice carrying a lacZ insertion mutation of the c-kit oncogene, which undergo the zygotic transfer of RNA molecules and inheritance of a white-tail phenotype (Rassoulzadegan et al. 2006).

In social invertebrates, the production of contrasting adult morphologies as well as different reproductive and behavioral systems is critical to their social organization and division of labor (Evans and Wheeler 1999, 2001; Hartfelder and Engels 1998). Recent studies suggest that nutritional effects on epigenetic mechanisms mediate this type of phenotypic plasticity. For example, fertile queens and sterile workers are alternative forms of the adult female honeybee (*Apis mellifera*) that develop from genetically identical larvae following differential feeding with royal jelly – a protein-rich substance secreted from glands on the heads of worker bees. A larva destined to become a queen is fed large quantities of royal jelly inside a specially constructed compartment called a queen cup. The queen is then fed royal honey exclusively for the rest of her life. Findings from recent studies suggest that royal jelly silences Dnmt3, an enzyme involved in genome-wide gene silencing in the newly hatched larvae, with effects on the larval developmental trajectory (Kucharski et al. 2008). Kucharski et al. 2008 showed that Dnmt3 siRNA-treated individuals emerged as queens with fully developed ovaries and a larger abdomen for egg laying, whereas the nontreated control larvae expressing Dnmt3 developed into the default sterile worker variety, due to epigenetic silencing of the genes encoding the queen phenotype. These results suggest that DNA methylation can be differentially altered by nutritional input, and that the flexibility of epigenetic modifications supports shifts in developmental fates, with implications for reproductive and behavioral status. Of course, genome-wide DNA methylation mapping and gene expression profiling in both social and solitary insects would reveal the underlying molecular interactions supporting these complex evolvable biological systems.

Perhaps one of the best illustrations in mammals of how phenotypes can differ dramatically due to epigenetic variability alone is the yellow agouti ($A^{vy}$) mouse model. These mice have a mutation upstream from the Agouti locus, which involves the de novo retrotransposition of a long terminal repeat (LTR) transposable element (Morgan et al. 1999). This intracisternal A-particle (IAP) transposon provides an alternate promoter for the gene, which drives expression during the development of the hair and causes a yellow fur phenotype. However, this alternate promoter overexpresses the gene in other tissues, leading to the eventual development of obesity and insulin resistance (Morgan et al. 1999). Genetically identical littermates with this IAP transposon have different levels of activity of the alternate promoter, and as a consequence, different degrees of fur discoloration and metabolic consequences. Importantly, the stable interindividual phenotypic variation is associated with the degree of variable histone modification and stochastic 5mC patterns at six CpG dinucleotides within the 5′ LTR of the $A^{vy}$ IAP promoter sequence (Dolinoy et al. 2010). In yellow mice, the $A^{vy}$ IAP promoter LTR is hypomethylated and enriched for H3 and H4 di-acetylation (H3/H4Ac2), which is associated with enhanced $A^{vy}$ IAP promoter activation, leading to yellow fur and susceptibility toward obesity and tumorigenesis. Conversely, in pseudoagouti mice, the $A^{vy}$ IAP promoter LTR is hypermethylated and enriched for H4

tri-methylation of lysine 20 residues (H4K20me3), which is associated with Agouti gene silencing, brown fur, and protection from obesity and cancer in adulthood (Dolinoy et al. 2010).

Variations in parental investment in mammals, such as nutrient supply provided by the parent and behavioral interactions, also affect the development of defensive responses and reproductive strategies in the progeny (Denenberg 1999; Ottinger and Tanabe 1969; Ressler and Anderson 1973). By providing nutrition, the maternal nurturing influences growth and development of offspring health (Morgan et al. 1999). As diet-derived methyl donors and cofactors are necessary for the synthesis of SAM, which serves as the donor of methyl groups for DNA methylation, environmental factors that alter early nutrition and/or SAM synthesis can potentially influence adult metabolism via persistent alterations in DNA methylation (Dolinoy et al. 2006; Waterland et al. 2006a; Waterland and Jirtle 2003; Waterland et al. 2006b; Wolff et al. 1998). In $A^{vy}$ mice, maternal supplementation with methyl donors (Waterland and Jirtle 2003) or the isoflavinoid genistein (Dolinoy et al. 2006) – a phytoestrogen found in soy and present at high levels in infant formula – during gestation can produce offspring with increased methylation of transposable elements in or upstream of the agouti gene, respectively, decreased Agouti gene expression, brown fur, and a reduced risk of lifelong chronic disease.

In the rat, dams fed a diet low in one-carbon donors during pregnancy produce offspring with decreased DNA methylation and increased association of H3K9Ac at promoter regions of specific genes, including the glucocorticoid receptor (GR) exon $1_{10}$ and peroxisome proliferator-activated receptor-alpha (PPAR$\alpha$), and increased mRNA expression in the liver of juvenile (Lillycrop et al. 2005) and adult offspring (Lillycrop et al. 2007). These epigenetic changes and reduced DNMT1 expression were largely prevented by maternal folic acid supplementation during pregnancy (Lillycrop et al. 2005, 2007, 2008) as well as by increasing folic acid intake during the juvenile–pubertal period (Burdge et al. 2009). Together with the $A^{vy}$ mice findings, these data suggest that early life nutrition has the potential to influence epigenetic programming in the brain not only during early development but also in adult life, thereby modulating health throughout life.

From an evolutionary perspective, the effects of nutrient restriction may be in response to adaptive programs that sense the organisms' nutritional state in gestation, which results in tissue-specific chromatin remodeling (Ke et al. 2006; MacLennan et al. 2004; Sinclair et al. 2007). Indeed, the availability of dietary methionine and folate alter the parent-of-origin effects on the methylation status of imprinted genes [including insulin-like growth factor (IGF)-2] (Luedi et al. 2005; Waterland et al. 2006b) that mediate many of the actions of growth hormone on somatic growth and tissue maintenance (Feinberg 2007). Support of this phenomenon is evidenced by recent studies in nonhuman primates that show maternal nutrient deprivation during pregnancy produce organ- and gestational age-dependent perturbations in global DNA methylation levels in the kidney and forebrain of the developing fetus (Unterberger et al. 2009).

In humans, it has long been proposed (Hales and Barker 1992) that poor fetal and infant growth; and the subsequent development of disease later in life emerge from

nutritional programming early in life (Wells 2003). Epidemiological data reveal that cardiovascular and diabetes mortality in children can be influenced by the nutritional status of their parents and grandparents (Kaati et al. 2002). However, the most comprehensive study to date of adaptive transgenerational epigenetic effects in mammals is that of the maternally transmitted responses to stress in the rat. In particular, differential maternal behavior in the rat alters the epigenetic status of the promoter of the hippocampal GR, which is associated with stable individual differences on stress responsivity and cognitive and emotional development in the offspring.

## 11.4 Natural Variations of Maternal Care in the Rat and Phenotypic Variability in the Adult Offspring

Natural variations in maternal care in humans and nonhuman primates are also observed in rodents and similarly are associated with divergent behavioral phenotypes in the offspring. In the rat, naturally occurring variations in the frequency of maternal pup licking/grooming and arched-back nursing (LG-ABN) are associated with stable interindividual differences on stress responsiveness, emotionality, cognitive performance, and reproductive behavior in the adult offspring (Caldji et al. 1998; Francis et al. 1999; Liu et al. 1997; Myers et al. 1989; Stern 1997). In adulthood, the female offspring of mothers that exhibit increased levels of pup LG-ABN (i.e., High LG-ABN mothers) over the first week of life are themselves high in maternal LG-ABN behavior toward their pups and likewise, the offspring of Low LG-ABN mothers are low in maternal LG-ABN behavior toward their pups (Francis et al. 1999). Neonatal cross-fostering as well as postweaning environmental enrichment and impoverishment can reverse behavioral differences between female High and Low LG-ABN offspring on measures of maternal behavior and anxiety (Champagne and Meaney 2007). Furthermore, gestational stress during the third trimester reduces LG-ABN behavior in High LG-ABN mothers and the adult offspring resemble those of Low LG-ABN mothers on measures of anxiety and maternal behaviors, an effect which is sustained through the second and third litter (Champagne and Meaney 2006). These findings suggest that individual differences in stress reactivity and maternal care are transmitted across generations through a behavioral mode of transmission linked to variations in maternal behavior, which serves to enhance the capacity for defensive responses in the progeny toward increased sensitivity to future (stressful) environments.

### *11.4.1 Hypothalamic–Pituitary–Adrenal and Behavioral Responses to Stress*

The relationship between early life environment and health in the adult offspring is mediated by maternal influences on the development of neuroendocrine systems

that underlie hypothalamic–pituitary–adrenal (HPA) and behavioral responses to stress (Francis and Meaney 1999; Heim et al. 2000; Nemeroff 1996; Repetti et al. 2002; Seckl and Meaney 1993; Sroufe 1997). Exposure to different levels of maternal LG-ABN during the postnatal period is associated with HPA blunting and changes in forebrain GR expression levels that persist into adulthood (Francis et al. 1999; Liu et al. 1997). The magnitude of the HPA response to stress is a function of the neural stimulation of hypothalamic corticotropin-releasing factor (CRF) release. This activates the pituitary–adrenal system as well as modulatory influences, as evidenced by glucocorticoid (GC) negative feedback in the hippocampus that inhibits CRF synthesis and release, dampening HPA responses to stress (De Kloet et al. 1998). GCs bind to GR to mediate the peripheral stress response and feedback to the CNS to modulate further activation of the HPA response (Herman et al. 2003).

The adult offspring of High LG-ABN mothers show increased hippocampal GR mRNA expression, enhanced feedback sensitivity to GCs, decreased hypothalamic CRF mRNA expression, and consequently more modest ACTH and cortisol stress responsivity, and reduced fearful behavior under conditions of stress in comparison with adult animals reared by Low LG-ABN dams (Caldji et al. 1998; Francis et al. 1999; Liu et al. 1997; Weaver et al. 2004, 2006, 2007). Interestingly, removing the difference in hippocampal GR levels erases the effects of early experience on HPA responses to stress in adulthood (Meaney et al. 1989), demonstrating that the difference in hippocampal GR expression serves as a mechanism for the effects of early experience on the development of individual differences in HPA responses to stress (Meaney 2001). Likewise, impairments to HPA axis regulation and an increase in anxiety-related behavior were observed in mice with forebrain-specific disruption of the GR gene and loss of hippocampal GR expression (Boyle et al. 2006). Furthermore, the adult offspring of the gestationally stressed High LG-ABN mothers have reduced hippocampal GR protein expression (Weaver et al. unpublished data) and resemble those of low LG-ABN mothers on behavioral measures of anxiety and maternal behavior (Champagne and Meaney 2006).

### *11.4.2 Maternal Effects and the Emerging Importance of the Epigenome*

The observations so far suggest that the developing rodent forebrain is vulnerable to tactile stimulation provided by the mother during the first week of life and that different frequencies of LG-ABN presented during this period help to program neurodevelopment with long-lasting consequences on hippocampal GR function and HPA responses to stress. Cross-fostering paradigms show direct effects of maternal care on the behavioral and neuroendocrine responses to stress, and therefore, support an epigenetic mechanism (Francis et al. 1999; Liu et al. 1997). In accordance with this hypothesis, we found differences in 5mC patterns and chromatin

H3K9Ac status in the exon $1_7$ promoter, an upstream regulatory region that regulates the expression of the coding regions of the GR gene (McCormick et al. 2000) in the hippocampus of the offspring of High and Low LG-ABN mothers (Weaver et al. 2004). These group differences emerge over the first week of life, remain stable into adulthood, and are reversed by cross-fostering (Weaver et al. 2004). More specifically, our data suggest that an epimutation within the transcription factor nerve growth factor-inducible protein-A (NGFI-A) response element in the GR exon $1_7$ promoter alters NGFI-A binding and might explain the sustained effect of maternal care on hippocampal GR expression and HPA responses to stress (Weaver et al. 2007).

The ability of maternal behavior to affect several behavioral phenotypes in the offspring, including maternal care, provides a mechanism by which acquired and stable behavioral traits can be propagated across generations through epigenetic modifications of DNA and chromatin structure. Support of this idea is evidenced by the increased hypothalamic CRF and GR exon $1_7$ promoter methylation, decreased central CRF and GR expression, and increased HPA responsivity in adult mice born to gestationally stressed dams (Mueller and Bale 2008). On the other hand, brief ($\leq$15 min) periods of handling of daily for the first weeks of life (which increases maternal LG-ABN behavior) is associated with increased repressor neuron-restrictive silencer factor (NRSF) and decreased hypothalamic CRF promoter expression in the adult offspring (Korosi et al. 2010). Within the CRF promoter is a NRSF response element (Seth and Majzoub 2001). Increased NRSF binding and recruitment of additional repressor complexes to the CRF promoter might explain the sustained effect of handling on hypothalamic CRF expression (Zheng et al. 2009).

Together with our findings, these data suggest that the enzymes required for DNA methylation are involved in programming of neuroendocrine function and behavior, and may also contribute to the timing and sensitivity of the neonate to maternal adversity during pregnancy and lactation.

### *11.4.3 From Maternal Care to Chromatin Plasticity and Beyond*

We propose that maternal behavior stimulates a signaling pathway that activates specific transcription factors, directing the epigenetic machinery (chromatin and DNA modifying enzymes) to exact targets within the genome. Our in vivo and in vitro studies suggest that maternal LG-ABN in early life elicits a thyroid hormone-dependent increase in serotonin (5-HT) activity at 5-$HT_7$ receptors, subsequent activation of cAMP and cAMP-dependent protein kinase A (PKA) accompanied by the recruitment of the transcription factor NGFI-A. NGFI-A, in turn, recruits CBP (which has HAT activity) (Chawla et al. 1998; Meaney et al. 1987, 2000; Weaver et al. 2007; Yu et al. 2004) and the demethylating enzyme MBD2b (Carvin et al. 2003) to the GR exon $1_7$ promoter (McCormick et al. 2000). Our data suggest that CBP activity increases H3K9Ac, opening the chromatin structure enabling the binding of both NGFI-A and MBD2b proteins simultaneously

to the same GR exon $1_7$ promoter sequence (Weaver et al. unpublished data). MBD2b demethylates the NGFI-A response element, allowing stable binding of NGFI-A to its cognate binding site resulting in an increase in levels of hippocampal GR expression in the neonatal offspring. NGFI-A discriminates between the methylated and unmethylated GR exon $1_7$ promoters and selectively activates the unmethylated sequences. Therefore, the different methylation states of the GR exon $1_7$ promoter from the offspring of High and Low LG-ABN results in different levels of hippocampal GR expression in the adult offspring. These data chart a course through which maternal behavior results in epigenetic modification of a specific gene in the brain. Interestingly, although MBD2-deficient mice are viable, the postpartum MBD2-null mothers were significantly slower at retrieving pups to their nests in comparison with the wild-type dams (Hendrich et al. 2001). This suggests that MBD2 might also have an important role in the behavioral transmission of epigenetic modifications across generations by the mother. Indeed, we have previously shown that this behavioral transmission is associated with cytosine methylation of the estrogen receptor (ER)-α 1b promoter and ER-α expression in the medial preoptic (MPOA) area of female offspring (Champagne et al. 2003, 2006). Notably, maternal care influences the maternal behavior of female offspring, an effect that also appears to be related to epigenetic regulation of endocrine function, providing a mechanism for transgenerational inheritance of maternal behavior from mother to offspring. As environmental stressors influence the nature of maternal behavior, maternal care remains a key mediator of epigenetic programming of neurodevelopment, and in turn, the expression of biological defense systems that respond to environmental adversity.

Similar processes at comparable epigenetic labile regions could explain why the adult offspring of High and Low LG-ABN dams exhibit wide spread differences in hippocampal gene expression and cognitive function (Weaver et al. 2006). For example, the adult offspring of Low LG-ABN mothers show enhanced binding of MECP2 to the BDNF promoter in the hippocampus (Weaver et al. unpublished data), decreased hippocampal BDNF mRNA and protein expression, reduced hippocampal neuronal survival, reduced hippocampal synaptogenesis, and synaptic plasticity (Bredy et al. 2003a; Liu et al. 2000; Weaver et al. 2002). Consequently, these offspring perform worse in tests of spatial learning and object recognition by comparison with adult animals reared by High LG-ABN dams (Bredy et al. 2003b; Liu et al. 2000). This is consistent with recent studies demonstrating that exposure of infant rats to stressed caretakers that predominately displayed abusive behaviors (e.g., dragging and rough handling) produces offspring with increased BDNF IV promoter methylation and decreased in forebrain BDNF mRNA expression throughout life, with evidence of transgenerational inheritance of these traits in the abused female offspring (Roth et al. 2009). Central infusion of the DNA methylation inhibitor zeburaline increases forebrain BDNF mRNA expression in the abused offspring to levels comparable with the nonabused offspring. Interestingly, the effect of maternal care on cognitive function in the offspring of Low LG-ABN mothers is largely reversed with peripubertal exposure to an enriched environment (Bredy et al. 2003b, 2004; Champagne et al. 2008), implying that

epigenetic labile regions in the rat brain remain environmentally responsive well beyond the perinatal period.

### *11.4.4 Evidence of Epigenetic Reprogramming in the Adult Brain*

Although the majority of epigenetic programming is thought to occur early in postnatal life, it is possible to reverse these patterns through social experience, diet, or pharmacological intervention (Fischer et al. 2007; Weaver et al. 2004, 2005). The potential for epigenetic programs to be reversed has several important implications, especially for interventions aimed at improving behavioral outcomes and cognitive performance in children born into adversity (Olds et al. 1998, 2004a, b).

Animal models suggest that there is the potential for phenotypic plasticity in response to social experience during adolescence and adulthood. In rodents, social isolation during the postweaning period is associated with decreased forebrain expression of 5-HT receptor subtypes and increased anxiety-like responses, and is attenuated by antidepressant treatment (Bibancos et al. 2007; Heritch et al. 1990). Similarly, adult rodents exposed to a single stressful agonistic behavioral encounter (social defeat) show prolonged increased CRF expression and HPA responses to stress (Buwalda et al. 1999; Cooper and Huhman 2007). Social avoidance behavior that was induced by chronic social defeat stress given in adulthood coincided with demethylation of the CRF gene and increased hypothalamic CRF mRNA transcript expression (Evan et al. 2010). Conversely, juvenile environmental enrichment in rats is associated with increased hippocampal NGFI-A and GR expression (Olsson et al. 1994), reduced basal coticosterone levels, and a reduced HPA response to stress (Belz et al. 2003). Long-term stress in the adult has been shown to result in hippocampal cell loss (Anderton 1997), promoting the notion that stress in early life might also alter hippocampal neuron structure and function permanently (Kerr et al. 1991; Sapolsky 1985). Remarkably, environmental enrichment increases hippocampal H3 and H4 acetylation and enhances cognitive performance in juvenile rodents that have selective loss of hippocampal neurons (Fischer et al. 2007).

We therefore examined the effects of pharmacological intervention on brain gene expression and physiological and behavioral responses to stress within the context of tactile stimulation in early life. Central infusion of the HDAC inhibitor (HDACi) trichostatin A (TSA) in the adult offspring of Low LG-ABN mothers increased the H3K9Ac, cytosine demethylation, NGFI-A binding, and GR exon $1_7$ promoter activation and reduced the HPA responses and anxiety-related behavior to levels comparable with those observed in the offspring of High LG-ABN dams (Weaver et al. 2004, 2006). Conversely, chronic central infusion of adult offspring of High or Low LG-ABN mothers with the dietary amino acid L-methionine, the precursor of SAM (Cantoni 1975; Mudd and Cantoni 1958) and inhibitor of demethylation (Pascale et al. 1991), increased the DNA methylation within the NGFI-A-binding site. This resulted in reduced NGFI-A binding to the exon $1_7$ promoter selectively in

the offspring of High LG-ABN dams, removing group differences in both hippocampal GR expression and HPA responses to stress (Weaver et al. 2005). The idea that DNA methylation patterns remain dynamic in adulthood is supported by recent work in rats receiving hippocampal-dependent contextual fear conditioning training (Miller et al. 2008; Miller and Sweatt 2007). Herein, TSA likely targets the class I HDAC2, as HDAC2 deficiency in mice results in increased synapse number and memory facilitation, similar to HDACi treatment (Guan et al. 2009). Together, these data suggest that the machinery required for de novo DNA methylation or demethylation remains present and responsive to cellular signaling cascades in the mature mammalian brain. Indeed, gene expression profiling of hippocampal tissue from the adult offspring of High and Low LG-ABN mothers reveal specific effects on the hippocampal transcriptome (Weaver et al. 2006).

## 11.5 Interindividual Differences in Human Behavior and Health

Studies in humans suggest that the forebrain GR function is complicit in the regulation of the HPA axis and the development of affective disorders (DeRijk and Sternberg 1997; Holsboer 2000; Invitti et al. 1999). This raises the question of whether epigenetic modification in response to early environmental conditions can explain the effects of early infant adversity on adult health in humans (for detailed review see Weaver 2009). Interestingly, during their lifetime, monozygotic twins increasingly differ in their epigenotype (lifelong drift) (Fraga et al. 2005), which might explain the frequent discordance of neuropsychiatric disorders such as schizophrenia and bipolar disorder (Kato et al. 2005). Although higher epigenetic discordance in fraternal (dizygotic) twins can result from differences in DNA sequence, recent in silico single nucleotide polymorphism (SNP) analyses together with animal studies favor epigenomic differences in the zygotes (Kaminsky et al. 2009). A recent review by Schlinzig et al. (2009) found that global DNA methylation levels in cord white blood cells are higher in newborns delivered by Caesarean section (CS) than those delivered by normal vaginal delivery. Although it is currently unknown how gene expression is affected in this case, individuals born by CS have been reported to face an increased risk for common diseases later in life (Cardwell et al. 2008; Hakansson and Kallen 2003). These studies raise the possibility that suboptimal epigenetic modifications arise over time resulting in late onset mental pathologies.

Indeed, aberrant gene transcription resulting from altered epigenetic regulation is associated with cognitive defects in several progressive pathologies including Alzheimer's disease (AD), schizophrenia, and depression. Increased expression of presenilin 1 (PS1), a member of the γ-secretase complex, correlates with PS1 promoter hypomethylation in postmortem brain samples from AD patients, and with increased β-amyloid formation in vitro (Scarpa et al. 2003; Wang et al. 2008). Down regulation of reelin, a glycoprotein involved in neuronal migration during development and cognitive functions in adults, and of the glutamate decarboxylase

that catalyzes GABA synthesis ($GAD_{67}$), are associated with promoter hypermethylation in postmortem samples of schizophrenic patients (Abdolmaleky et al. 2005; Guidotti et al. 2000; Impagnatiello et al. 1998).

Consistent with this hypothesis, ribosomal RNA (rRNA) promoter methylation (Brown and Szyf 2007, 2008) was shown to be increased in suicide victims who were victims of abuse during childhood compared with controls (McGowan et al. 2008), suggesting a reduced capacity for protein synthesis in suicide brains (Brown and Szyf 2007, 2008). Protein synthesis has long been known to be required for associative learning to consolidate into long-term memory (Agranoff et al. 1967), which involves epigenetic regulation (Korzus et al. 2004), and a decline in cognitive plasticity is commonly observed with age (Kadar et al. 1990). More recently, it was found that the expression levels of DNMT enzymes are altered in suicide brains and specific genes are aberrantly silenced by DNA methylation (Poulter et al. 2008). For example, the GABA-A $\alpha 1$ receptor subunit (Poulter et al. 2008) and BDNF exon IV (Keller et al. 2010) promoter regions are both hypermethylated and their gene expression reduced in the forebrains of depressed patients who committed suicide in comparison with controls, further suggesting a link between mental disease and abnormal methylation. These findings suggest that a shift in the steady-state balance between DNA methylating and demethylating machinery might influence specific neural pathways and account for interindividual differences in emotional reactivity and mental health in humans.

The effects of tactile stimulation through mother–infant interactions on interindividual differences in cognitive development and stress responses in rodents is supported by work in humans (Feldman et al. 2002) and nonhuman primates (Harlow and Zimmermann 1959). This raises the question of whether comparable epigenetic labile regions to the GR exon $1_7$ promoter exist in the human genome. Alignment of splice sites reveals that the distally located exon 1F promoter of human type II GR (hGR, OMIM +138040; NR3C1) shows high homology to the GR exon $1_7$ promoter in the rat, and contains an NGFI-A-binding site (Turner and Muller 2005). Interestingly, studies in healthy human subjects show that CpG methylation patterns of conserved transcription factor binding sites on the NR3C1 exon 1F promoter are both stochastic and unique to the individual (Turner et al. 2008). Furthermore, neonatal methylation at the 5′ CpG dinucleotide within the NGFI-A-binding site on the NR3C1 exon 1F promoter has been suggested as an early epigenetic marker of maternal mood and risk of altered HPA function in infants 3 months of age (Oberlander et al. 2008). Although future studies are required to examine the functional consequence of the methylated 5′ CpG dinucleotide, these findings are consistent with our studies in the neonate and adult offspring of Low LG-ABN mothers that show hypermethylation of the 5′ CpG dinucleotide within the NGFI-A-binding site on the exon $1_7$ promoter, decreased GR expression and increased HPA responsivity (Weaver et al. 2004). In support of this paradigm, a recent study shows that the NGFI-A-binding sites on the NR3C1 exon 1F promoter are hypermethylated in the hippocampus of suicide victims with a history of childhood abuse and mRNA transcript expression from the exon 1F

promoter is decreased by comparison to controls (victims of sudden, accidental death with no history of abuse) (McGowan et al. 2009), suggesting that the transmission of vulnerability for depression from parent to offspring could occur through the epigenetic modification of genomic regions that are implicated in the regulation of stress responses.

## 11.6 Concluding Remarks

The dynamic interdependence of an individual's genome, lifestyle, and health later in life has lead to the realization that the epigenome is essentially the study of gene–environment interactions. Sensory input during early development plays an important role in brain development with long-term consequences on brain functioning in adulthood. The studies presented in this chapter provide support for the idea that mother–offspring interactions early in life enhance the capacity for defensive responses in the progeny by programming emotional, cognitive, and endocrine systems toward increased sensitivity to adversity, and that these programs can be transmitted across generations via an epigenetic mode of inheritance involving maternal behavior, which in turn is responsive to changes in environmental stimuli (biotic, physical, and social). However, epigenetic reprogramming of distinct patterns of gene expression can take place at several points throughout life in response to developmental, physiological, psychological, pathological, and/or environmental cues.

These findings are restricted to the study of a single promoter in only one gene in one brain region; at this time, these results might be best thought of as a proof of principle. The degree to which this mechanism generalizes to environmental programming in other systems remains to be determined, and may well reveal an alternative mechanism for programming of the epigenome. The challenge will be to find comparable epigenetic labile regions. Genome-wide methodologies (epigenomics, metabolomics, proteomics, transcriptomics, etc.) of epigenetic changes assessing different regions and cell types in the brain are necessary. The examination of genotype–epigenotype–environment interactions from a developmental perspective has broad ranging implications for our understanding social, physiological, and pathological processes and their interrelations. Accordingly, we are only beginning to understand the mechanisms whereby early life experience suppresses or enhances expression of adaptive phenotypic behaviors throughout life.

## 11.7 Competing Interests Statement

The author declares that he has no competing financial interests.

**Acknowlegments** I would like to thank Dr. Shelley E. Brown for her helpful comments and numerous constructive suggestions throughout the preparation of this manuscript.

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# Chapter 12
# Histone Deacetylase Inhibitors: A Novel Therapeutic Approach for Cognitive Disorders

**Viviane Labrie**

**Abstract** Epigenetic mechanisms have a central role in regulating gene expression and are capable of influencing complex cognitive functions. In particular, acetylation of histone proteins is an epigenetic modification involved in mediating synaptic plasticity, learning, and memory. Emerging evidence indicates that increased histone acetylation through the inhibition of histone deacetylases (HDACs) can facilitate the formation of long-term memories in preclinical studies. Moreover, HDAC inhibitors have been reported to ameliorate cognitive deficits in animal models relevant to neurodegenerative and neurodevelopmental disorders. HDAC inhibitors have also been found to enhance the extinction of learned behaviors, including drug-seeking behaviors. Consequently, HDAC inhibition may be a useful approach in the treatment of a wide range of disorders characterized by cognitive dysfunction. Future HDAC-based pharmacotherapies will benefit from a greater understanding of different HDAC isoforms and the molecular pathways specifically involved in inducing cognitive enhancement.

**Keywords** Drug addiction · Histone deacetylase (HDAC) inhibitors · Memory · Neurodegenerative disorders · Neurodevelopmental disorders · Synaptic plasticity

## 12.1 Introduction

Epigenetics, a cellular mechanism once considered to be stable after development, has now been found to be a dynamic process that occurs in fully differentiated, postmitotic cells of the central nervous system in response to environmental signals (Borrelli et al. 2008; Graff and Mansuy 2008; Feinberg 2007). Epigenetic mechanisms regulate the structure of the chromatin through posttranslational modifications

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_12,
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of histone proteins and covalent DNA modifications. Alterations in chromatin structure changes the accessibility of DNA to regulatory proteins, resulting in changes in gene transcription. Changes in gene expression and subsequent protein synthesis affect synaptic function and morphology, which is essential for many cognitive processes, including learning and memory (Costa-Mattioli et al. 2009; Loebrich and Nedivi 2009). In this manner, epigenetic mechanisms act as a means of translating external stimuli into modifications of gene expression and neural activity that affect cognitive function and behavioral outcome.

One of the most extensively studied cognitive processes is memory formation. Recent studies have demonstrated that chromatin modifications, especially histone acetylation, are involved in synaptic plasticity and memory formation (Fischer et al. 2007; Levenson et al. 2004; Korzus et al. 2004; Guan et al. 2002). In general, histone acetylation is associated with transcriptional activation and is regulated by the balance between histone acetyltransferases (HAT) and histone deacetylases (HDAC). Increased histone acetylation has been shown to facilitate learning behaviors (Guan et al. 2009; Fischer et al. 2007; Levenson et al. 2004). This has led a number of researchers to investigate the potential of HDAC inhibitors to improve memory, particularly in diseases characterized by cognitive impairments. Non-selective HDAC inhibitors have demonstrated ameliorative effects in preclinical models relevant to Alzheimer's disease, Huntington's disease, Rubinstein–Taybi syndrome, and other cognitive disorders (Fischer et al. 2007; Kilgore et al. 2010; Ferrante et al. 2003; Alarcón et al. 2004; Korzus et al. 2004). HDAC inhibitors have also been found to augment extinction, a learned adaptive response capable of benefiting treatments for persistent maladaptive behaviors observed in many psychiatric illnesses and drug addiction (Lattal et al. 2007; Bredy and Barad 2008). These promising therapeutic effects have stimulated considerable interest in determining the HDAC isoforms, gene targets, and molecular pathways involved in mediating cognitive improvement. Consequently, the development and analysis of HDAC inhibitors is a rapidly expanding field. This chapter reviews recent insights about the contributions of chromatin acetylation to physiological and pathological cognitive functioning, and the application of HDAC inhibitors for the treatment of neurodegenerative, neurodevelopmental, and psychiatric disorders.

## 12.2 Histone Modifications

Histone proteins are components of the chromatin architecture that function to compress genomic DNA within the nucleus and are intimately involved in regulating gene expression by controlling access and signaling to the transcriptional machinery (for review, see Kouzarides 2007; Berger 2007). Complexes of DNA and histones form the nucleosome, the basic unit of the chromatin, in which 146 base pairs of DNA is wrapped around an octamer of core histone proteins: H2A, H2B, H3, and H4. Histones are small, conserved, and highly basic proteins composed of a globular domain and an amino-terminal tail that protrudes from the

surface of the nucleosome. Histone tails are the site of numerous posttranslation modifications, including acetylation, phosphorylation, methylation, sumoylation, ubiquitination, and ADP-ribosylation. Patterns of histone modifications have been proposed to encode information, since these modifications affect chromatin structure and correspond to differing degrees of transcriptional activation or repression. These patterns are often referred to as the "histone code" (Strahl and Allis 2000) and can be altered in response to external stimuli (Borrelli et al. 2008; Graff and Mansuy 2008).

Acetylation of histones has been correlated with active gene expression (for review, see MacDonald and Howe 2009; Choi and Howe 2009). Histone acetylation occurs at lysine residues on histone tails and neutralizes existing positive charges, which weaken the histone–DNA interaction. This relaxes the chromatin structure and allows transcription factors to interact with their target DNA. The steady-state levels of histone acetylation are determined by the balance between the activity of HATs and HDACs. HAT enzymes catalyze the addition of acetyl groups to lysine residues within histones, thereby facilitating gene transcription. Removal of acetyl groups is the responsibility of HDACs. Elevated HDAC activity shifts the chromatin to a more condensed conformation, repressing gene expression. Interestingly, recent gene-array findings suggest that the effect of HDAC activity on gene expression is not global. Less than 20% of genes demonstrate an altered expression level in response to HDAC inhibition *in vitro* (Li and Li 2006; Iacomino et al. 2001; Tabuchi et al. 2006; Glaser et al. 2003; Chiba et al. 2004) and *in vivo* (Shafaati et al. 2009; Bosetti et al. 2005; Covington et al. 2009).

The superfamily of HDACs is divided into four main classes: I, II, III, and IV (for review, see Kazantsev and Thompson 2008; Carey and La Thangue 2006). Class I and II are the most well studied in the nervous system and contain $Zn^{2+}$-dependent active sites. Class I HDACs consist of HDAC1, HDAC2, and HDAC3, which are ubiquitously expressed and HDAC8, which is primarily restricted to smooth muscle cells. Class I HDACs are predominately localized in the nucleus. Class II HDACs are further divided into two subgroups: class IIa (HDAC4, HDAC5, HDAC7, and HDAC9) and class IIb (HDAC6 and HDAC10). Class II enzymes share considerable structural and sequence homology, and can shuttle between the cytoplasm and nucleus. Class III HDACs, known as the sirtuins (SIRT1–7), require nicotinamide adenine dinucleotide ($NAD^+$) for their enzymatic activity. Class IV is represented by a single member, HDAC11, which is principally located in the nucleus of cells.

With the exception of HDAC8, all HDAC isoforms exist in large multisubunit complexes and associate with other proteins or HDACs for optimal enzymatic activity (Balasubramanian et al. 2009). For example, HDAC4, HDAC5, and HDAC7 are unable to deacetylate histones in isolation and require interaction with HDAC3 to be active (Fischle et al. 2002). Also, in the cell, HDAC3 is complexed with NCOR/SMRT, which improves HDAC3 deacetylation activity (Guenther et al. 2001). HDAC1 and HDAC2 are associated with mSin3, NuRD, and CoREST in the cell, forming complexes that are recruited to gene promoters by DNA binding proteins, which may contribute to gene-specific transcriptional

regulation (Laherty et al. 1997; Zhang et al. 1999; Wen et al. 2000). Thus, the diversity of isoforms, multiprotein complexes, and cofactors makes development of selective HDAC inhibitors a formidable challenge.

## 12.3 HDAC Inhibitors

The development of new HDAC inhibitors is currently undergoing a period of sudden growth in the pharmaceutical industry due to the potential application of these compounds as anticancer therapies and the emerging possibility that HDAC inhibitors may be useful in treating neurological and psychiatric illnesses. HDAC inhibitors can be classified into four major categories based on chemical structure (1) the short-chain fatty acids, such as sodium butyrate, valproate, and phenylbutyrate; (2) the hyroxamic acids, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA); (3) the benzamides, such as MS-275, 4b, and 106; and (4) the cyclic peptides, including apicidin and depsipeptide (Thomas 2009; Ma et al. 2009). In cancer, HDAC inhibitors can suppress tumor cell proliferation, induce cell differentiation, and upregulate crucial genes associated with anticancer effects (Carey and La Thangue 2006; Carew et al. 2008). As a consequence, various HDAC inhibitors have entered phase I and II clinical trials for cancer treatment, and have so far demonstrated promising antitumor activity for several types of cancers (Ma et al. 2009). However, the goal of reversing or impeding pathophysiological processes in disorders of the central nervous system is fundamentally different than the objectives of anticancer therapies. Harnessing the therapeutic potential of HDAC inhibitors for neurological and psychiatric disorders requires stable and bioavailable compounds capable of crossing the blood–brain barrier to reach diseased brain tissues. Some of the most commonly investigated HDAC inhibitors in the central nervous system include sodium butyrate, valproate, phenylbutyrate, TSA, and SAHA. These compounds are predominately broad-spectrum inhibitors, targeting many HDAC isoforms in both class I and II. Valproate affects only class I HDAC isoforms, however, valproate is also known to have many additional targets, and so the contribution of HDAC inhibition to the therapeutic effects of this drug is unclear. Certain HDAC inhibitors, such as SAHA and sodium butyrate display greater potencies in inhibiting class I HDACs than class II enzymes (Kilgore et al. 2010). Indeed, SAHA displays a high affinity for class I HDACs, with effective concentrations in the low nanomolar range (Kilgore et al. 2010; Khan et al. 2008).

The design of class- and isoform-specific HDAC inhibitors has been highly difficult due to the evolutionarily conserved architecture of HDAC active sites. However, attempts to improve selectivity by exploiting subtle differences in active site components, including the capping group, linker region, and metal-binding domains, have had some success (Bieliauskas and Pflum 2008). Selective compounds targeting exclusively HDAC1 or HDAC8 in the class I category and HDAC4 or HDAC6 in the class II category have been developed (Perez-Balado et al. 2007; Kozikowski et al. 2008; Krennhrubec et al. 2007; Bieliauskas and Pflum 2008).

Certain compounds are specific for a limited number of HDAC isoforms, such as SHI-1:2 inhibitors, which demonstrate an intrinsic activity against HDAC1 and HDAC2 that is at least a 100-fold greater than for other HDACs (Methot et al. 2008). Furthermore, a family of pimelic diphenylamide HDAC inhibitors demonstrating class I specificity with no class II activity have been created (Chou et al. 2008). These compounds also show a more than 15-fold selectivity for HDAC3 in comparison with HDAC1 and HDAC2 (Chou et al. 2008). Other compounds selective for class I or class II HDAC enzymes have been generated (Hamblett et al. 2007; Jones et al. 2008; Ontoria et al. 2009; Bieliauskas and Pflum 2008). As these compounds have just emerged, reports on their effects in the central nervous system are very limited. However, these compounds will be important in determining the roles of individual HDACs in physiological and disease processes in the brain. In addition, isoform- and class-selective HDAC inhibitors may more effectively targeting the pathophysiological disturbances involved in cognitive disorders.

An important caveat in interpreting all studies using HDAC inhibitors is that modifications of the epigenetic histone code may not be the exclusive or even primary mechanism underlying the effects of HDAC inhibitors. It is becoming increasingly apparent that HDAC enzymes not only target histones, but also regulate the acetylation of a variety of nonhistone proteins (Glozak et al. 2005; Lin et al. 2006; Carey and La Thangue 2006). This includes transcription factors, signal transduction mediators, cytoskeletal proteins, and several metabolic enzymes (Glozak et al. 2005; Lin et al. 2006; Carey and La Thangue 2006). Although histones are the most thoroughly studied substrate of HDACs, lysine residues on many nonhistone proteins are deacetylated by HDACs. Consequently, HDAC enzymes are more accurately considered as "lysine deacetylases". Changes in the acetylation of nonhistone proteins can potentially influence stability, localization, protein dimerization, and binding activity of these proteins and may be an additional method by which HDACs mediate transcriptional regulation (Caron et al. 2005; Levy et al. 2004; Gronroos et al. 2002; Wang et al. 2001). Therefore, the behavioral effects of HDAC inhibitors may be due to alterations in the acetylation of a diverse number of intracellular targets.

Another key point concerning the effects of HDAC inhibition is that patterns of histone acetylation are intricately linked to other epigenetic modifications. Indeed, extensive cross-talk occurs between different histone modifications and DNA methylation (Kouzarides 2007; Latham and Dent 2007). This involves multiple feed-forward and feed-back mechanisms within- and between-nucleosomes, allowing epigenetic modifications to act in concert (Kouzarides 2007; Latham and Dent 2007). For example, methylated DNA, a genomic sequence with a transcriptionally repressive epigenetic mark, can recruit methyl-binding proteins (MBDs), such as methyl-CpG-binding protein 2 (MeCP2) (Meehan et al. 1992; Hendrich and Bird 1998). MBDs associate with histone methyltransferases (HMTs) and HDACs that methylate and deacetylate histones, respectively, further silencing gene expression (Jones et al. 1998; Fuks et al. 2003). In addition, class I HDAC inhibitors valproate and MS-275 have been found to induce histone acetylation and lower DNA methylation in the frontal cortex of mice with hypermethylated DNA (Dong et al. 2007).

Similarly, the pan-HDAC inhibitor TSA has been shown to induce global and gene-specific DNA demethylation in human cancer cells (Ou et al. 2007). Thus, histone acetylation is unlikely to be the only epigenetic modification altered by HDAC inhibitors. Also, due to the complexity of the interactions, classification of epigenetic mechanisms as gene activating or suppressing is likely an oversimplification.

## 12.4 Regulation of Histone Acetylation in Learning and Memory

Histone acetylation has been implicated in many complex behaviors, particularly learning and memory. Memory is described as an array of processes employed by the brain for the long-term storage of information. The formation of long-term memories entails lasting changes in gene expression. Indeed, studies have demonstrated that the establishment of long-term memories requires the engagement of many signal pathways, changes in gene transcription, and de novo protein synthesis (Costa-Mattioli et al. 2009; Loebrich and Nedivi 2009).

Memory formation has been found to induce increases in histone acetylation in the hippocampus. The hippocampus is a brain region of particular importance to the formation and retrieval of memories (Kim and Fanselow 1992; Szapiro et al. 2002). In rodents, learning and memory can be investigated using a number of well-established hippocampus-dependent tests, including spatial mazes, contextual fear conditioning, and novel object recognition. In contextual fear conditioning, animals learn to associate a novel context with an aversive stimulus, and a single training session is sufficient to induce the formation of long-term memories. Animals exposed to a contextual fear conditioning paradigm demonstrated a significant increase in histone acetylation (Levenson et al. 2004). The increase in acetylation was transient and found at 1 h, but not 24 h after training, and only in histone H3, but not H4 (Levenson et al. 2004). The solely transient and H3-specific change in acetylation observed may be the result of using pan-specific antibodies that probe multiple acetylation sites. However, the finding that memory consolidation correlates with increased histone acetylation has been reproduced in a number of other learning paradigms using rodents (Fischer et al. 2007; Vecsey et al. 2007; Fontan-Lozano et al. 2008; Levenson et al. 2004) and invertebrates (Federman et al. 2009). Interestingly, transient elevations in histone acetylation associated with memory formation were found to be mediated by *N*-methyl-D-aspartate (NMDA) receptor-dependent synaptic transmission and the mitogen-activated protein kinase (MAPK) signaling cascade (Levenson et al. 2004). NMDA receptor activation and MAPK signaling are cellular processes critically involved in the formation of long-term memories in the hippocampus (Atkins et al. 1998).

Consistent with the idea that histone acetylation has a functional role in learning and memory, mice with a disruption in HAT activity display memory impairments. CREB-binding protein (CBP) is an enzyme involved in recruiting other components of the transcriptional machinery and contains endogenous HAT activity

(Bannister and Kouzarides 1996). Several genetically-modified mice with impaired CBP function have been developed (Alarcón et al. 2004; Wood et al. 2005, 2006; Oike et al. 1999). These models consistently demonstrate that abnormal CBP function leads to deficits in a number of memory tests, including novel object recognition, spatial memory, and contextual and cued fear conditioning (Alarcón et al. 2004; Wood et al. 2005, 2006; Oike et al. 1999). However, it is possible that memory abnormalities in CBP transgenic animals are not only the result of altered HAT activity, since CBP interacts with multiple transcriptional regulators (Janknecht 2002). To address this, mice expressing a CBP transgene with an inactivated HAT domain ($CBP^{HAT}$) were generated (Korzus et al. 2004). Expression of $CBP^{HAT}$ was spatially restricted to forebrain neurons and temporally restricted using the tetracycline system, thereby avoiding potential developmental abnormalities. $CBP^{HAT}$ expression in adult mice decreased histone acetylation and produced impairments in spatial and recognition memory that could be normalized by transgene suppression. In support of these findings, studies examining other HAT enzymes, E1A-binding protein (p300), and p300/CBP-associated factor (PCAF) have also shown a role for these HATs in memory processes (Oliveira et al. 2007; Maurice et al. 2008). Transgenic mice expressing an inhibitory truncated version of p300 that lacks HAT activity were demonstrated to have deficits in long-term recognition and contextual fear memory (Oliveira et al. 2007). These studies indicate that HAT activity contributes to memory formation, and the role of HAT in learning and memory may be mediated through histone acetylation. However, like HDACs, HATs are also known to target several nonhistone proteins. HAT-induced acetylation of transcription factors, such as p53, GATA-1, and Myo-D, has been shown to alter their DNA binding affinity and enhance transcription (Boyes et al. 1998; Gu and Roeder 1997; Polesskaya et al. 2000).

Histone acetylation may also facilitate synaptic plasticity. Synaptic plasticity refers to the ability of synapses to undergo activity-dependent changes in strength. At a cellular level, memory formation is dependent on synaptic plasticity (Bliss and Collingridge 1993; Costa-Mattioli et al. 2009). In *Aplysia*, histone acetylation was found to be differentially regulated by opposing forms of synaptic plasticity (Guan et al. 2002). The sensorimotor synapse of *Aplysia* demonstrates two major forms of long-term synaptic plasticity: long-term facilitation (LTF), which is a lasting enhancement in synaptic transmission, and long-term depression (LTD), which is a persistent decrease in synaptic transmission. LTF was accompanied by an increase in binding of the HAT CBP to the CAAT/enhancer-binding protein (C/EBP) gene promoter with a concurrent increase in histone H3 and H4 acetylation at the same promoter (Guan et al. 2002). In contrast, LTD led to a decrease in histone acetylation at the C/EBP promoter that was mediated at least in part by HDAC5 (Guan et al. 2002). Changes in histone acetylation have also been observed during the induction of synaptic plasticity in mammalian systems. Long-term potentiation (LTP) reflects an increase in synaptic transmission in response to high-frequency stimulation. LTP requires the activation of NMDA receptors and engages the MAPK signaling cascade (Kauer et al. 1988; Kelleher et al. 2004). Activation of NMDA receptors in the hippocampus was shown to increase histone H3 acetylation and this effect was

blocked by inhibition of MAPK signaling (Levenson et al. 2004). MAPK activation in the hippocampus also augmented histone H3 acetylation (Levenson et al. 2004). The MAPK signaling pathway may promote histone acetylation through CBP HAT activity, as the MAPK cascade is known to lead to the phosphorylation and activation of CREB, which recruits CBP for subsequent transcriptional activation (Impey et al. 2002). Genetically-manipulated mice with deficient CBP function demonstrate impaired synaptic plasticity in the late-phase of LTP (L-LTP), which is known to require gene transcription (Alarcón et al. 2004). Administration of the HDAC inhibitor SAHA to hippocampal slices of CBP-deficient mice significantly improved L-LTP (Alarcón et al. 2004). Therefore, these studies indicate that histone acetylation may be involved in the synaptoplastic processes underlying memory formation.

## 12.5 Modulation of Learning and Memory by HDAC Inhibitors

Since memory formation is associated with an increase in histone acetylation and can be disrupted by reduced HAT activity, it may follow that compounds that increase histone acetylation effectively enhance memory formation. To test this directly, a number of studies have investigated the effects of HDAC inhibitors on long-term memory formation in normal animals. Systemic administration of an HDAC inhibitor, such as TSA, sodium butyrate, valproate, or SAHA, improved long-term memory in tests of contextual fear conditioning (Levenson et al. 2004; Fischer et al. 2007; Guan et al. 2009; Bredy and Barad 2008), cued fear conditioning (Bredy and Barad 2008), spatial learning in a water maze (Fischer et al. 2007), eyeblink conditioning (Fontan-Lozano et al. 2008), and object recognition (Fontan-Lozano et al. 2008) in normal rodents. Direct infusion of TSA into the hippocampus or amygdala has also been found to enhance fear-associated memories (Vecsey et al. 2007; Yeh et al. 2004). Some studies did not observe a memory improvement in wild-type animals following HDAC inhibitor administration in certain behavioral tasks (Kilgore et al. 2010; Vecsey et al. 2007). However, the degree of memory enhancement observed is likely determined by the properties of the HDAC inhibitor investigated (for example, the binding affinity and HDAC isotype selectivity) and by the dosing strategy, learning procedure, genetic background, and age of the animals employed.

Supporting the facilitatory effects of HDAC inhibitors on memory formation, these compounds have been demonstrated to augment synaptic plasticity and synaptogenesis. In the normal rodent hippocampus, induction of LTP was significantly enhanced by HDAC inhibition, using TSA, sodium butyrate, or SAHA (Levenson et al. 2004; Alarcón et al. 2004). In addition, LTP induced in the amygdala was increased by TSA (Yeh et al. 2004). Recently, chronic administration of SAHA in wild-type mice was found to enhance dendritic spine density and synapse numbers (Guan et al. 2009), which are cellular processes known to contribute to synaptic plasticity and memory formation (Yang et al. 2009; Bourne and Harris 2010).

HDAC inhibitors have also been shown to transform a learning event that would not normally result in long-term memory into an event that is remembered long-term. Mice exposed to a novel object for a short period of time will not remember this object after 24 h (Stefanko et al. 2009; Fontan-Lozano et al. 2008). However, mice treated with an HDAC inhibitor such as sodium butyrate or TSA displayed a lasting memory for the object that persisted after 24 h and 7 days (Stefanko et al. 2009; Fontan-Lozano et al. 2008). Similarly, in invertebrates, a weak training protocol that is insufficient to establish lasting memories was shown to potentiate long-term memory in animals treated with sodium butyrate or TSA (Federman et al. 2009). Administration of TSA to sensorimotor neurons of *Aplysia* resulted in a switch from short- to long-term facilitation of synaptic transmission (Guan et al. 2002). In mouse hippocampal slices, early-LTP (E-LTP), which does not require transcription or translation, was transformed into a transcription-dependent long-lasting form of LTP (Vecsey et al. 2007). Furthermore, HDAC inhibition decreased the threshold for learning-induced changes in histone acetylation and expression of memory-associated genes. Mice treated with sodium butyrate or TSA displayed a greater increase in histone H3 acetylation in the hippocampus and perirhinal cortex after exposure to an object recognition task (Fontan-Lozano et al. 2008) and contextual fear conditioning paradigm (Vecsey et al. 2007). In addition, HDAC inhibitors increased the expression of genes specifically associated with learning and memory following a learning task (Fontan-Lozano et al. 2008; Vecsey et al. 2007). This is consistent with studies demonstrating that HDAC inhibitors do not act globally, but can regulate a smaller subset of genes in the brain (Bosetti et al. 2005; Covington et al. 2009). Overall, these findings indicate that HDAC inhibitors can generate long-term memories under conditions that are typically insufficient to induce lasting memories, and that these facilitatory effects may be mediated by changes in histone acetylation and transcription of genes involved in memory formation.

Recently, HDAC inhibitors have been demonstrated to improve the extinction of memories. Extinction is regarded as a distinct form of learning that acts to suppress, but not erase, previously established memories (Davis et al. 2006). Like other forms of learning, extinction has been shown to depend on an intricate regulatory network of signaling cascades, gene transcription, and protein synthesis (Davis et al. 2006). Extinction of conditioned fear has been associated with changes in histone acetylation (Bredy et al. 2007). Following extinction, the promoter region of brain-derived neurotrophic factor (BDNF), a gene implicated in memory and synaptic plasticity, was found to have altered histone H3 and H4 acetylation in the prefrontal cortex of mice (Bredy et al. 2007). Moreover, systemic or intrahippocampal administration of sodium butyrate, TSA, or valproate in normal mice was found to enhance the extinction of contextual and cued fear (Lattal et al. 2007; Bredy and Barad 2008). Valproate was shown to improve the effects of a weak extinction protocol and induced similar changes in histone H4 acetylation in the BDNF promoter region as a strong extinction protocol (Bredy et al. 2007). Thus, in addition to having therapeutic potential for diseases involving impaired memory, these results suggest that HDAC inhibitors may be

useful in treating diseases characterized by persistent maladaptive memories, such as posttraumatic stress disorder and drug addiction.

## 12.6 HDAC Inhibitors as a Potential Treatment for Cognitive Disorders

Since HDAC inhibitors benefit memory processes in normal mice, several studies have investigated the potential of these compounds in ameliorating cognitive symptoms in disease models. Cognitive dysfunction is a central component of many neurological and psychiatric illnesses, and often includes disturbances in learning and memory. Consequently, the capacity of HDAC inhibitors to improve learning and memory has been examined in several animal models of cognitive impairment, including models of neurodegenerative diseases, neurodevelopmental disorders, and drug addiction.

### *12.6.1 Neurodegenerative Diseases: Recovering Lost Memories*

Marked neuronal atrophy accompanied by a progressive decline in memory is a prominent characteristic of neurodegenerative disorders such as Alzheimer's disease (Heese and Akatsu 2006). Several mouse models with extensive neurodegeneration have been developed and recent studies have begun to explore the potential of HDAC inhibitors to improve memory formation in these models (Eriksen and Janus 2007). In CK-p25 transgenic mice, postnatal induction of p25, a protein implicated in various neurodegenerative diseases, specifically in the forebrain resulted in cyclin-dependent kinase 5 (Cdk5) hyperactivation (Fischer et al. 2005). This led to deficits in spatial and associative fear memory, along with severe synaptic and neuronal loss in forebrain structures such as the hippocampus (Fischer et al. 2005). Chronic administration of the HDAC inhibitor sodium butyrate prior to a learning task was shown to effectively enhance associative and spatial memory, and also increased synaptic connectivity (Fischer et al. 2007). Furthermore, the possibility that HDAC inhibitors may reestablish access to memories that had become lost or inaccessible as a result of neurodegenerative processes was also investigated using the CK-p25 transgenic mice (Fischer et al. 2007). Mice were initially trained in fear conditioning and spatial learning procedures. Following a rest period to allow long-term memory consolidation, induction of p25 was initiated, causing brain atrophy and neuronal loss. After p25 induction, animals were given chronic injections of sodium butyrate or vehicle, and then fear-associated and spatial memories were assessed. Remarkably, chronic HDAC inhibitor administration led to the recovery of memories that would otherwise be inaccessible. Thus, HDAC inhibitors were found to improve memory formation and restored the

capacity for memory recall in a mouse model of neurodegeneration. Interestingly, the permissive effects of HDAC inhibitors on memory in CK-p25 transgenic mice were mimicked by environmental enrichment (Fischer et al. 2007). Environmental enrichment, a combination of exercise and cognitive training, is a well known but poorly understood means of enhancing memory in rodents and humans. It has been reported to delay the onset and development of memory deficits and neuropathologies associated with Alzheimer's disease (Berardi et al. 2007; Lazarov et al. 2005; Jankowsky et al. 2005). CK-p25 mice subjected to environmental enrichment demonstrated a similar increase in histone acetylation and in memory formation and retrieval as mice treated with an HDAC inhibitor (Fischer et al. 2007). This suggests that environmental enrichment and HDAC inhibitors may facilitate memories through a similar mechanism that could involve histone acetylation.

HDAC inhibitors have been tested in other mouse models relevant to Alzheimer's disease. The accumulation of neurotoxic amyloid plaques and neurofibrillary tangles in the brain are neuropathological hallmarks of Alzheimer's disease. Genetic mutations in amyloid precursor protein (APP) and presenilin-1 (PS1) have been implicated in plaque formation in early-onset cases of Alzheimer's disease (Selkoe 2001). Overexpression of human APP and PS1 mutations in mice induced amyloidosis and prominent cognitive deficits (Jankowsky et al. 2004; Savonenko et al. 2005). HDAC inhibitors, including valproate, sodium butyrate, and SAHA, were shown to reverse impairments in contextual fear memory in mice expressing mutant APP and PS1 transgenes (Kilgore et al. 2010). In addition, treatment with an HDAC inhibitor allowed the newly formed memories to remain stable, as the memories continued to be present after 2 weeks (Kilgore et al. 2010). Nicotinamide, a competitive inhibitor of class III HDACs, was also found to prevent impairments in spatial memory, object recognition, and fear conditioning in genetically-modified mice that develop both amyloid plaques and neurofibrillary tangles (Green et al. 2008). In these mice, nicotinamide reduced intracellular aggregates of a specific phospho-species of tau associated with microtubule depolymerization and neurofibrillary tangles (Green et al. 2008). Finally, in mice treated with kainic acid, a neurotoxic agent that induces hippocampal neurodegeneration and cognitive impairments, HDAC inhibitors sodium butyrate and TSA were found to restore short- and long-term memory in an object recognition task (Fontan-Lozano et al. 2008). Thus, these animal models indicate that HDAC inhibitors may be suitable for treating cognitive dysfunction in Alzheimer's disease. However, the mechanisms underlying Alzheimer's disease are far more complex than those reproduced in these animal models, and evidence has argued that Alzheimer's disease is associated with an increase and decrease in histone acetylation (Cao and Sudhof 2001; Marambaud et al. 2003; Kim et al. 2004, 2007; Rouaux et al. 2003; Saura et al. 2004). Conceivably, both types of changes in histone acetylation co-occur in Alzheimer's disease, and vary depending on brain structure, cell type, and genomic region. Consequently, it remains to be determined whether HDAC inhibitors will indeed provide therapeutic benefits to Alzheimer's patients.

Alterations in histone acetylation have also been implicated in Huntington's disease (HD), a neurodegenerative disorder characterized by cognitive impairments,

mood disturbances, and motor deficits (Sadri-Vakili and Cha 2006). HD is caused by a CAG repeat expansion in the coding region of the *huntingtin* gene, producing mutant huntingtin protein with a lengthened polyglutamine tract (Sadri-Vakili and Cha 2006). The polyglutamine extension was found to directly bind and inhibit the activity of the HAT domain of CBP and PCAF (Steffan et al. 2001). This inhibitory interaction was demonstrated to lead to a reduction in histone H3 and H4 acetylation in cultured *Drosophila* cells that was reversed by administration of sodium butyrate, TSA, or SAHA (Steffan et al. 2001). In mouse models of HD, treatment with HDAC inhibitors, such as phenylbutyrate, sodium butyrate, 4b, and SAHA, have been shown to attenuate neuronal atrophy, increase motor function, and extend survival (Ferrante et al. 2003; Hockly et al. 2003; Thomas et al. 2008; Gardian et al. 2005). This was accompanied by an increase in the acetylation of histones and nonhistone proteins associated with neurotoxicity and HD pathology (Ferrante et al. 2003; Hockly et al. 2003; Gardian et al. 2005; Sadri-Vakili et al. 2007; Dompierre et al. 2007). The effects of HDAC inhibitors on cognitive performance in animal models of HD have not been reported, likely due to the confounding effects of motor deficits in these models. However, clinical trials investigating the efficacy and safety of the HDAC inhibitor phenylbutyrate as a treatment for HD have been initiated (study ID: R01NS45242), and will shed light on the capacity of these compounds to improve the cognitive symptoms of HD.

### *12.6.2 Neurodevelopmental Disorders*

Rubinstein-Taybi syndrome (RTS) is a rare developmental disease that is characterized by mental retardation and skeletal abnormalities (Hallam and Bourtchouladze 2006). RTS is caused by mutations in the gene encoding CBP (Petrij et al. 1995; Blough et al. 2000). Recent evidence indicates that RTS symptoms are also caused by mutations in the gene encoding p300 (Roelfsema et al. 2005; Bartholdi et al. 2007). As mentioned earlier, CBP and p300 have HAT activity and function as transcriptional coactivators that recruit other components of the transcriptional machinery. Mouse models with deficient CBP or p300 display cognitive and physiological deficits associated with RTS (Alarcón et al. 2004; Korzus et al. 2004; Wood et al. 2005; Oliveira et al. 2007). Memory impairments induced by a CBP deficiency were demonstrated to be reversed by systemic or intraventricular injections of the HDAC inhibitors TSA and SAHA (Alarcón et al. 2004; Korzus et al. 2004). In addition, HDAC inhibition ameliorated synaptic plasticity in CBP-deficient mice, as SAHA reversed impaired L-LTP (Alarcón et al. 2004). At the molecular level, reduced HAT activity and histone acetylation in CBP transgenic mice was increased by HDAC inhibitors (Alarcón et al. 2004; Korzus et al. 2004). Thus, HDAC inhibitors constitute a promising therapeutic approach for the cognitive features of RTS.

Fragile X syndrome (FXS) is a common heritable disease in which patients exhibit a wide range of neurological disturbances, including cognitive deficits,

autism, seizures, peripheral neuropathy, and abnormal autonomic function (Garber et al. 2008). FXS is caused by an expanded repeat of CGG trinucleotides at the 5′ end of the fragile X mental retardation 1 (*FMR1*) gene (Verkerk et al. 1991; Pieretti et al. 1991). The trinucleotide expansions cause an increase in DNA methylation at the *FMR1* gene promoter, reducing the translational of the fragile X mental retardation protein (FMRP) (Pieretti et al. 1991; Godler et al. 2010). FMRP is a selective RNA-binding protein that regulates mRNA transport to neuronal dendrites and local translation, affecting synaptic plasticity and neuronal maturation (Weiler et al. 1997). Treatment of lymphoblastoid cells from FXS patients with demethylating agents such as 5-aza-2-deoxycytidine reactivated *FMR1* gene expression and restored normal levels of *FMR1* mRNA and protein (Chiurazzi et al. 1998). Administration of the HDAC inhibitors 4-phenylbutyrate, sodium butyrate, or TSA in combination with 5-aza-2-deoxycytidine was far more effective, indicating a synergistic effect between histone hyperacetylation and DNA demethylation (Chiurazzi et al. 1998). Accordingly, subsequent studies demonstrated that DNA methylation is tightly coupled to histone acetylation and histone methylation at the *FMR1* gene (Coffee et al. 2002; Tabolacci et al. 2008). Recently, inhibitors of class III HDACs, nicotinamide and splitomicin, were shown to induce *FMR1* gene reactivation and the acetylation of histones at the 5′ end of the *FMR1* gene in cells derived from FXS patients (Biacsi et al. 2008). These findings support the need for further investigation about the ability of HDAC inhibitors to improve the symptoms of FXS.

### *12.6.3 Drug Addiction*

Exposure therapy is one of the most effective forms of psychotherapy and is commonly used to treat anxiety syndromes and substance abuse (Hofmann 2007; Marlatt 1990). Exposure therapy is based on the concept of extinction. For drug addictions, treatment involves prolonged and repeated exposures to drug-associated environmental cues without access to the substance of abuse. Consequently, this form of treatment allows patients to diminish conditioned responses elicited by the drug-associated cues (Heather and Bradley 1990; O'Brien et al. 1990). Recent evidence indicates that pharmacotherapies capable of enhancing extinction in rodents have the potential to improve the effectiveness of exposure therapy in humans (Davis et al. 2006; Norberg et al. 2008).

Drug-seeking behaviors in mice can be measured using the conditioned place paradigm (CPP). In this task, animals learn to pair the effects of an addictive substance with a distinct environment. Extinction is induced by repetitive exposures to the drug-associated context without reinforcement. Extinction of cocaine-seeking behaviors measured in the CPP was enhanced by treatments with the HDAC inhibitor sodium butyrate (Malvaez et al. 2009). Reinstatement of drug-seeking behaviors after the extinction sessions can be induced by administering a drug prime. Sodium butyrate was found to significantly attenuate the

reinstatement of cocaine-seeking behaviors (Malvaez et al. 2009). Thus, HDAC inhibition enhanced the rate of extinction and its persistence, suggesting that HDAC inhibitors may facilitate the effects of exposure therapy for substance abuse disorders in the clinic.

## 12.7 Mechanisms by Which HDAC Inhibitors Facilitate Cognitive Function

One of the major open questions that arise from studies investigating the therapeutic utility of HDAC inhibitors is: What are the mechanisms by which HDAC inhibitors enhance cognitive function and synaptic plasticity? To address this question, Guan et al. (2009) recently performed a series of experiments investigating the HDAC family members specifically involved in memory formation. Initially, SAHA, an HDAC inhibitor that primarily targets class I HDACs (HDAC1, HDAC2, and HDAC3 in the brain) and HDAC6, was confirmed to enhance memory formation in normal mice. The possibility that SAHA induces memory improvements by reducing HDAC6 activity was eliminated by demonstrating that a selective HDAC6 inhibitor, WT-161, did not increase memory in normal mice. The contributions of HDAC1 and HDAC2 to memory formation were investigated using genetic knockout or overexpression mouse models. HDAC2 overexpression was found to decrease dendritic spine density, synapse number, and synaptic plasticity. Moreover, elevated HDAC2 levels attenuated memory formation in behavioral measures of contextual and cued fear conditioning, spatial long-term memory, and spatial working memory. In contrast, overexpression of HDAC1 did not alter memory or synaptic function. Genetic inactivation of HDAC2 was found to increase synaptogenesis, synaptic plasticity, context- and tone-associated memories, and spatial working memory. If HDAC inhibitors induce memory enhancements via HDAC2, then it would be expected that HDAC inhibition would readily reverse the learning impairments in mice overexpressing HDAC2, but have no effect in mice lacking HDAC2 activity. Indeed, SAHA enhanced associative fear memories and completely abrogated the decrease in spine density and synapse abnormalities in HDAC2-overexpressing mice. Conversely, SAHA did not affect associative fear memory, spine density, synapse numbers, or synaptic plasticity in HDAC2 knockout animals. This suggests that HDAC2 is the major target of SAHA in facilitating memory formation. Further studies are necessary to determine whether these effects are reproduced using other HDAC inhibitors. Importantly, mice lacking HDAC2 did not display gross changes in neuronal morphology or behavioral deficits, indicating that HDAC2 may be safely targeted without causing obvious disruptions in neuronal physiology and brain function. Thus, selective HDAC2 inhibitors may be a suitable treatment for cognitive disorders.

HDAC2 has been proposed to mediate changes in memory formation and synaptic function by binding to the regulatory element of memory-associated genes and suppressing their expression (Guan et al. 2009). HDAC2 was found to be enriched

at the promoter regions of *BDNF*, *CBP*, cAMP responsive element binding protein 1 (*Creb1*), early growth response factor 1 (*Egr1*), FBJ osteosarcoma oncogene (*Fos*), neuritin 1 (*Nrn1*), neurexin 3 (*Nrxn3*), and NMDA receptor subunits (Guan et al. 2009). All these genes are implicated in synaptic plasticity and remodeling or regulated by neuronal activity (Loebrich and Nedivi 2009). In addition, HDAC1 was demonstrated to be more enriched at the promoter region of activity regulated cytoskeletal-associated protein (*Arc*), a gene associated with learning and memory processes (Guan et al. 2009). This indicates that although HDAC2 appears to be the principal isoform negatively regulating memory formation, HDAC1 may also provide important contributions. Interestingly, S-nitrosylation of HDAC2 has been found to release HDAC2 from the promoter of *Egr1*, a memory-associated gene (Nott et al. 2008). S-nitrosylation is induced by nitric oxide, a free radical molecule that can be synthesized in response to neuronal activation (Nott et al. 2008). Reduced HDAC2 activity following S-nitrosylation or in HDAC2 knockout mice was correlated with an increase in the acetylation of histones in synaptic plasticity genes, such as *BDNF*, *Egr1*, *Fos*, and the NMDA receptor subunit *GLUR1* (Guan et al. 2009; Nott et al. 2008). Accordingly, the expression of these genes was also elevated (Guan et al. 2009; Nott et al. 2008). Thus, HDAC inhibitors may improve memory formation by decreasing the activity of HDAC2 (and other pertinent isoforms), resulting in an increase in histone acetylation and a subsequent elevation in the transcription of genes involved in synaptic plasticity and synaptogenesis.

HDAC inhibitors have also been reported to enhance memory formation and synaptic plasticity through CREB- and CBP-dependent transcriptional activation (Vecsey et al. 2007). Genetic elimination of CREB function was shown to attenuate the enhancement in contextual fear memory and hippocampal LTP induced by the HDAC inhibitor TSA. Transgenic mice carrying a version of CBP lacking the CREB-binding domain were found to be resistant to the facilitatory effects of TSA on hippocampal LTP. Furthermore, HDAC inhibitors were shown to augment the expression of CREB-targeted genes after a contextual fear conditioning procedure. This suggests that in order for HDAC inhibitors to improve memory, simply inducing a histone hyperacetylated state is not sufficient and activation of the CREB and CBP transcriptional complex may also be required.

Other nonhistone targets have been implicated in the memory enhancement elicited by HDAC inhibitors. Nuclear actor-kappa-B (NF-κB) is a transcription factor that positively regulates the expression of genes and influences synaptic plasticity, learning, and memory (Hoffmann et al. 2006; Romano et al. 2006). Acetylation of the p65 subunit (RelA) of the NF-κB complex enhances its DNA binding affinity and transcriptional activity (Chen et al. 2002). p65 acetylation was found to be transiently increased in the rodent amygdala following associative fear learning (Yeh et al. 2004). Treatment with the HDAC inhibitors TSA or sodium butyrate prior to the learning task was shown to significantly prolong the expression of p65 and increase its DNA binding affinity (Yeh et al. 2004). Eliminating p65 accessibility using a decoy κB DNA segment attenuated the TSA-induced improvement in associative fear conditioning (Yeh et al. 2004). Furthermore, many other transcription factors and nonhistone proteins are

regulated by changes in acetylation, including p53, FOXO1, p300, Hsp90, and tubulin (Glozak et al. 2005; Lin et al. 2006). Consequently, acetylation of nonhistone substrates likely contributes to the mechanism by which HDAC inhibitors mediate memory improvements.

## 12.8 Conclusions and Future Directions

To date, pan-HDAC inhibitors have demonstrated a capacity to improve memory formation in a wide diversity of models relevant to neurodegenerative and neurodevelopmental diseases (Fischer et al. 2007; Kilgore et al. 2010; Ferrante et al. 2003; Alarcón et al. 2004; Korzus et al. 2004). HDAC inhibitors have also been shown to facilitate the extinction of persistent and maladaptive behaviors (Lattal et al. 2007; Bredy and Barad 2008), indicating that HDAC inhibitors may enhance the effects of exposure therapy used to treat anxiety syndromes and addiction. HDAC inhibitors therefore demonstrate promising effects for the treatment of many cognitive disorders involving deficits in learning and memory. However, there remain many open questions critical to our understanding of the effects of HDAC inhibitors on complex cognitive functions in the brain. First, determining the effects of HDAC inhibitors on different cell types and structures of brain will be necessary to better predict the consequences of HDAC inhibition. Indeed, evidence demonstrates that expression patterns of many HDAC isoforms is different among brain regions (Broide et al. 2007), suggesting that the outcome of distinct HDAC inhibitors may vary depending on the targeted isoforms. Second, we are still far from understanding the signaling cascades and gene expression changes that are necessary and sufficient to mediate the cognitive improvements induced by HDAC inhibitors. Third, how are HDAC inhibitors capable of exerting transcriptional control on a select subset of genes? A recent high-resolution mass spectrometry study identified 3,600 lysine acetylation sites on 1,750 proteins and found that HDAC inhibitors only upregulated about 10% of all acetylation sites (Choudhary et al. 2009). Moreover, understanding the relative contributions of histone and nonhistone proteins will be central to determining the mechanisms mediating the effects of HDAC inhibitors. Finally, knowledge of the HDAC isoforms involved in eliciting the cognitive improvements will lead to the development of selective HDAC-based therapies. In this regard, genetic animal models targeting specific HDAC isoforms indicate that HDAC2 inhibitors may be particularly useful in treating cognitive dysfunction (Guan et al. 2009). Currently, the development of isoform- and class-selective HDAC inhibitors is undergoing a rapid expansion. Investigations of these selective HDAC inhibitors will uncover the role of HDAC subtypes in normal physiology and disease, and will facilitate rational drug design based on pathophysiological insight. In addition, improving selectivity will likely limit the number of untoward side effects in the clinical setting. Thus, selective HDAC inhibitors represent a fundamentally novel approach for treating cognitive disorders that may deliver symptomatic improvements with fewer adverse effects.

**Acknowledgments** V.L. is supported by a Canadian Institutes of Health (CIHR) fellowship.

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# Chapter 13
# Epigenetic Mechanisms of Memory Consolidation

**Marcel A. Estevez and Ted Abel**

**Abstract** The requirement of gene transcription for memory consolidation has been established for several decades, but not until recently has it been recognized that epigenetic mechanisms play a role in the control of gene expression that leads to memory storage. Broadly speaking, these epigenetic mechanisms encompass changes in chromatin structure, such as posttranslational histone modifications and DNA methylation. Many studies have shown that histone acetylation is dynamically regulated after experience and that it acts as a permissive switch to allow gene transcription. These studies have established a dichotomy where permissive gene transcription results in memory enhancement and restrictive gene transcription in memory impairment. Other studies have focused on DNA methylation, specifically on the promoter region of genes, where it is associated with gene inactivation. Like histone acetylation, it has been shown that DNA methylation is regulated after experience, and can be dynamically regulated, although it is thought to be a more stable mark than histone acetylation. The relationship between levels of DNA methylation and memory impairment or enhancement is unclear and will require future study. Many other chromatin modifications play a role in experience-induced gene regulation, such as histone methylation and phosphorylation, as well as substitution by histone variants. Ultimately, these modifications in chromatin structure act in concert, in a potential combinatorial code, to regulate gene-specific transcription that results in memory consolidation.

**Keywords** Combinatorial chromatin code · DNA methylation · Electroconvulsive treatment · Histone acetylation · Histone methylation · Histone phosphorylation · Long-term memory · Long-term potentiation · Memory consolidation · Synaptic plasticity

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_13,
© Springer-Verlag Berlin Heidelberg 2011

Unlike most science jargon, *memory* is a common term used in everyday life. Nevertheless, *memory* is rather abstract and not uniformly defined. As scientists, we describe it as both a behavioral adaptation and information storage, which come as a result of experience. In terms of information storage, there are different stages for memory that have been described: *acquisition*, *consolidation*, and *retrieval*. Memory acquisition refers simply to learning, that is, the encoding of information after an experience. Memory retrieval is the remembering of the experience, which is where a behavioral adaptation can be measured. In between these two temporally spaced stages lies memory consolidation. Memory consolidation is the establishment of *long-term* memory, lasting days, weeks, or even years after acquisition. Somehow the coded information is stabilized into a more persistent state. Not all information learned is consolidated, however, as most experiences are not committed to long-term storage. This labile information is referred to as *short-term* memory, which can only be retrieved in a short span of time after acquisition.

The study of the underlying mechanisms of memory consolidation is a daunting task. Firstly, the code for memory is not understood. Secondly, several memory systems have evolved to store different types of information. For instance, reflex conditioning depends on cerebellar circuitry, conditioned emotional responses depend on amygdalar circuitry, and cognitive or relational learning depends on hippocampal circuitry (Rugg 1997). Furthermore, consolidation is not a single process but a family of processes, as there is a distinction between synaptic or cellular consolidation versus system consolidation. At the level of a synapse or cell, consolidation occurs in a matter of minutes or hours, whereas at a system level, consolidation occurs in the time frame of days or weeks. In the latter, it is believed that the circuitries of the brain that encode memory are reorganized and that the information storage is displaced from the circuitry where it was encoded, e.g., the hippocampus, to a more permanent site of retention, e.g., the neocortex (Dudai 2004).

Memory research has mostly focused on studying cellular correlates and molecular components in neurons that underlie memory storage via *synaptic* or *cellular consolidation*. Moreover, the study of memory has focused on declarative memory – the memory of facts and places that is dependent on hippocampus and cortex for consolidation and maintenance. One of the first steps in understanding the molecular composition of memory came in the 1960s and 1970s when several groups showed that protein synthesis inhibitors block formation of long-term, but not short-term, memory consolidation in fish and rodents (e.g., Agranoff et al. 1966; Barondes and Cohen 1967). These initial studies looked at discrimination tasks based on reward, which are dependent on hippocampus. Although the time window for protein synthesis inhibitor to have amnesic effects is variable (Gold 2006), these initial studies establish that protein synthesis is needed immediately after the learning experience.

Not surprisingly, transcription immediately after the learning experience is also required for memory consolidation (Squire and Barondes 1970; Neale et al. 1973) and also 3–6 h after training (Igaz et al. 2002). Seemingly paradoxically,

electroconvulsive stimulation, which causes increased transcription of immediate early genes (Saffen et al. 1988; Morgan et al. 1987), is an amnesic treatment. So, whereas long-term changes in synaptic plasticity and memory require transcription and translation of these early gene products, indiscriminate, mass neuronal transcriptional activation impedes memory formation. This suggests that some level of specificity of neuronal activity and consequent gene regulation is required for memory, while mass neuronal activity masks the storage of memory. Many of these early genes that are induced during learning or mass neuronal stimulation are transcription factors, which are then able to initiate another wave of transcription. The products of these transcriptional waves result in long-lasting changes in synaptic strength, which is widely understood as a cellular correlate of memory.

## 13.1 Long-Term Potentiation as a Correlate of Long-Term Memory

Activity-dependent changes in synaptic strength in the hippocampus are thought to underlie memory storage and the acquisition of learned behaviors. One intensely studied form of synaptic plasticity is long-term potentiation (LTP), a persistent, activity-dependent form of synaptic enhancement that is a model for certain types of long-term memory (Bliss and Collingridge 1993; Martin et al. 2000; Neves et al. 2008). LTP in the CA1 region of the hippocampus has distinct temporal phases (Nguyen and Woo 2003): a transient, early phase (E-LTP) that lasts 1–2 h and a late phase (L-LTP) that lasts for up to 8 h in hippocampal slices (Frey et al. 1993) and for days in the intact animal (Abraham et al. 1993). Both E-LTP and L-LTP in the CA1 region depend on the *N*-methyl D-aspartate (NMDA) receptor and on the activation of kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII), whereas L-LTP (but not E-LTP) shares with long-term memory a requirement for transcription and translation (Frey et al. 1988, 1993, 1996; Frey and Morris 1997; Huang and Kandel 1994, 1995; Huang et al. 1996; Nguyen et al. 1994; Nguyen and Kandel 1997; Nguyen and Woo 2003).

Studies in *Aplysia* and *Drosophila* have been important in establishing a role for the cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA) pathway in memory consolidation (Carew 1996). This pathway leads to activation by phosphorylation of the cAMP response element-binding protein (CREB), a transcriptional activator for many immediate early genes. It has been shown in these invertebrate models that not only does inactivation of CREB disrupt long-term memory, but CREB activation enhances it. The role for CREB in mammalian memory was later elucidated using a mouse genetic approach, where two CREB isoforms, namely α and δ, were deleted from the genome resulting in memory impairment (Bourtchuladze et al. 1994). Moreover, CREB overexpression also results in *enhanced* memory consolidation in rats (Josselyn et al. 2001). Together

with comparable data from invertebrate models, these studies demonstrate that CREB has a key role in modulating memory consolidation.

Considering the limited time window for protein synthesis and transcription inhibitors blocking memory consolidation, and also the short half-life of mRNA and protein, it is puzzling how the gene products made in this time window result in the long-lasting neuronal changes of memory consolidation. Certainly, these gene products must pave the way for mechanisms required to maintain memory. A widely accepted view is that these activity-dependent gene products result in lasting changes in synaptic morphology and strength. However, recent studies suggest that persistent changes in gene expression may also underlie the maintenance of memory.

Recently, much attention has shifted to the regulation of the state of *chromatin* for gene regulation during memory consolidation. Chromatin, the complex of genomic DNA with packaging proteins, exists in different states where genes or gene clusters are either expressed or repressed. Chromatin exists in different states, grossly identified as euchromatin and heterochromatin. The regulation of these states of chromatin has been studied to explain the regulation of gene expression in different cell types and the maintenance of chromosomal regions such as telomeres and centromeres. During cell differentiation, chromatin is silenced or activated to regulate the expression of genes according to a specific cell type. These chromatin states are considered extremely stable in terminally differentiated, postmitotic cells, accounting for the maintenance of the identity of the differentiated cell. However, it has become evident that some chromatin changes are quite dynamic in postmitotic cells, such as neurons and immune cells that undergo gene expression changes in response to experience (e.g., Borrelli et al. (2008) review epigenetic control in neuronal plasticity; Placek et al. (2009) review epigenetic control in CD4+ T cell activation). Chromatin regulation is possible through covalent modifications in nucleosomal proteins known as histones, and also the DNA itself. Some of these modifications are more labile, such as histone acetylation, while others such as DNA methylation are much more stable. Here, we focus on the role of these chromatin modifications during memory consolidation.

## 13.2 Histone Acetylation

Histones are basic proteins that are modified by acetylation, phosphorylation, methylation, ubiquitination, and sumoylation of their amino-terminal tails (Strahl and Allis 2000; Peterson and Laniel 2004). A groundbreaking finding in the field of memory was to show that inhibition of histone deacetylation *enhances* memory, which is reminiscent of the effect of CREB overexpression (Levenson et al. 2004; Fischer et al. 2007; Alarcón et al. 2004; Vecsey et al. 2007; Stefanko et al. 2009). Because the increase of acetylated histones in response to histone deacetylase inhibitor is global, and not cell- or gene-specific, it suggests that histone acetylation alone, unlike mass neuronal activity, is not sufficient for gene expression. Moreover, it suggests that the

specificity of neuronal activity confers memory consolidation, while histone acetylation is a gate for this to occur.

In the seminal study by Levenson et al. (2004), the authors use the contextual fear conditioning paradigm, a hippocampus-dependent association task, to investigate the role of histone acetylation in hippocampus-dependent memory. One of their major findings is that training in this association task leads to a significant increase in acetyl-H3 in area CA1 of the hippocampus, but not acetyl-H4. Also, pre-exposure to the context – which blocks the association of context and shock – does not result in increased acetyl-H3, but does increase acetyl-H4. Moreover, both phorbol ester and forskolin treatments, which activate protein kinase C (PKC) and protein kinase A (PKA), respectively, mimic this acetyl-H3 increase without acetyl-H4 increase, suggesting a role for these pathways in association-dependent histone acetylation. In a later study, they show that DNA methylation is required for the phorbol ester-induced H3 acetylation (Levenson et al. 2006). It should be noted, however, that global changes in acetylation may not correlate to gene-specific changes, as forskolin induces acetylation of histone H4, but not H3, in the *Bdnf* promoter IV in cultured neuroblastoma cells (He et al. 2010).

The MAPK pathway is necessary for the increase in acetyl-H3 (Levenson et al. 2004), and the study proposes that the PKA and PKC pathways converge upstream of the MAPK pathway. Another study by the same group also implicates the MAPK pathway in histone H3 phosphorylation, which is also induced by the contextual fear conditioning paradigm (Chwang et al. 2006). The work by Levenson et al. (2004) also shows that broad-spectrum HDAC inhibitors, namely sodium butyrate (NaB) and trichostatin A (TSA), enhance a form of hippocampal LTP. The LTP protocol that the authors use is two 100-Hz stimuli, spaced by 20 s. This LTP protocol is not particularly robust, and also not PKA-dependent (Abel et al. 1997), although it is sufficient to elicit a long-lasting potentiation of synaptic strength. Lastly, a major finding in the study is that sodium butyrate administered intraperitoneally is able to enhance long-term (24 h) associative memory (contextual fear conditioning) but not short-term (1 h) memory.

A study by Fischer et al. (2007) expands on this previous study by showing residue-specific histone acetylation and changes in synaptic morphology. In their study, they use a neurodegenerative disease mouse model and show that environmental enrichment rescues learning and memory deficits. Unlike the Levenson study, this study looks at histone acetylation and synaptic spine morphology after environmental enrichment and not after associative learning. The authors show a significant increase in acetylation of specific residues in histones H3 and H4, 24 h or 2 weeks after environmental enrichment. In the hippocampus, some increases return to baseline at 2 weeks (Ac-H3K14, Ac-H4K8, and Ac-H4K12), whereas some stay elevated after 2 weeks (Ac-H3K9 and Ac-H4K5) not only in hippocampus, but also in cortex, which is congruent with a role for the cortex in remote memory storage. Also, methyl-H3K4 is elevated at 24 h and at 2 weeks after enrichment in cortex only. Together with the previous studies, these findings suggest that environmental enrichment acts in a manner similar to histone deacetylase inhibitors to increase histone acetylation and enhance contextual memory and modulate synaptic morphology.

## 13.3 Histone Acetyltransferases and Histone Deacetylases

The study of the transcriptional coactivator, and histone acetyltransferase (HAT), CREB-binding protein (CBP) in memory consolidation came with the recognition of the importance of histone acetylation in memory, together with the established role for cAMP/PKA/CREB signaling in this process. Also, along with CBP, other factors with histone acetyltransferase activity such as p300, a CBP homologue, and p300/CBP-associated factor (PCAF) became targets of memory studies. CBP and p300 are large and widely expressed multidomain transcriptional coactivators with HAT activity. A role for CBP in synaptic plasticity first came from studies in *Aplysia* (Guan et al. 2002). The role of these transcriptional coactivators in cognition had already been recognized, as mutations in the human CBP or p300 genes map with Rubenstein–Taybi syndrome (RTS) (Petrij et al. 1995; Roelfsema et al. 2005). Different loss-of-function and dominant-negative CBP and p300 mouse models have been used to study the role of these factors in memory. One such study uses heterozygous *Cbp*-mutant mice, which express a truncated CBP protein that lacks the C-terminus, thus inhibiting CBP function and resulting in RTS-like phenotypes (Oike et al. 1999). This RTS mouse model has impaired long-term memory in passive avoidance, fear conditioning, and object recognition, demonstrating a role for CBP in memory consolidation (Bourtchuladze et al. 2003; Oike et al. 1999).

In a haploinsufficiency model of RTS carrying a single null allele of *Cbp* (Tanaka et al. 1997, 2000), deficits in long-term memory for object recognition and fear conditioning are observed, and these deficits are ameliorated by histone deacetylase (HDAC) inhibitor treatment (Alarcón et al. 2004). This is the first study to implicate CBP in HDAC inhibitor-mediated synaptic strengthening and memory enhancement. The authors show that this heterozygous null mutant mouse, $Cbp^{+/-}$, which only has one functional allele of *Cbp*, has deficits in long-term contextual associative memory and in hippocampal LTP. These memory and plasticity impairments are rescued by an HDAC inhibitor, suggesting that CBP-mediated histone acetylation is required for memory consolidation and LTP. They also show that the mice have a significant, constitutional 50% decrease of acetylated histone H2B, while there are no changes in the other core nucleosome histones (H2A, H3, and H4). It is interesting that the target of acetylation in these *Cbp* mutants (namely, H2B) is different than the target of acetylation induced by environmental enrichment (H3 and H4), associative learning (H3), or nonassociative context exposure (H4). This suggests that multiple histones are required to form a code for memory formation. This also highlights the difficulty in assessing a role for acetylation of a specific histone, or histone residue, at a whole genome scale as opposed to individual target genes.

These results suggest that CBP and its HAT activity have a role in long-term memory. However, the developmental abnormalities and broad effects of the *Cbp* mutants described above make it difficult to draw conclusions on an adult role for CBP. Moreover, these mutants still have a functional copy of the *Cbp* gene. To address these concerns, two studies using transgenic mouse models with regulated

expression of dominant-negative CBP constructs have confirmed a role for CBP in memory storage without confounding developmental abnormalities (Korzus et al. 2004; Wood et al. 2005). One of these constructs, CBPΔ1, lacks the HAT domain of CBP and has deficits in long-term contextual memory but not short-term contextual memory, implicating HAT activity of CBP in memory consolidation (Wood et al. 2005). Moreover, another *Cbp* mutant that lacks the ability to interact with phospho-CREB, $Cbp^{kix/kix}$, also has a contextual memory deficit without a developmental phenotype (Wood et al. 2006).

The study by Vecsey et al. (2007) uses the $Cbp^{kix/kix}$ mutant to implicate the CREB–CBP interaction in HDAC inhibitor-mediated memory enhancement. The authors show that a mutant CREB mouse lacking two isoforms of the transcription factor (α and δ), $Creb\alpha\delta^{-/-}$, (Bourtchuladze et al. 1994) and $Cbp^{kix/kix}$ (Wood et al. 2006), both of which exhibit deficits in HDAC inhibitor-mediated memory and LTP enhancements. This finding suggests that the recruitment of CBP by CREB is necessary for HDAC-mediated memory and LTP enhancement.

The role of p300 in memory was also addressed by using similar approaches as in the case for CBP. Two published studies look at different p300 mutant mouse models in contextual memory. A study by Oliveira et al. (2007) uses a transgenic dominant-negative approach, equivalent to the CBPΔ1 mutant, p300Δ1, which is expressed in forebrain neurons to block p300 function. Mice expressing this dominant negative, which lacks the HAT domain of the protein, have deficits in long-term contextual memory but not in short-term memory. Viosca et al. (2010) use a heterozygous null, $p300^{+/-}$, to assess the role of p300 in RTS etiology. While these mice have some developmental abnormalities, they did not exhibit deficits in contextual or spatial memory consolidation. The mice did, however, show impairment in relearning a spatial memory (water maze transfer task – where the mice have to relearn a new position for the platform in the Morris water maze). This learning deficit reflects a higher order role for p300 in plasticity or reorganization of a previous memory. This deficit could be at either a cellular- or system-level consolidation.

Another study related to HAT activity suggests a role for PCAF in memory acquisition, but not consolidation (Duclot et al. 2010). This study uses PCAF null mice in a spatial working memory task.

While the roles of CBP and p300 had been widely studied in the context of histone acetylation and memory, the target of the HDAC inhibitors implicated in memory enhancement had not been identified. HDACs comprise a large superfamily with mainly three classes. Both class I and class II HDACs are NAD-independent, whereas class III HDACs, also called sirtuins, are NAD-dependent. Both TSA and NaB inhibit largely class I and II HDACs, but within these are many HDACs that are expressed in the brain.

A recent study identifies HDAC2, a class I HDAC, as the main target of HDAC inhibitor-mediated memory enhancement (Guan et al. 2009). The study uses both *Hdac2* knockouts, as well as HDAC1 and HDAC2 overexpressors (OE). While the HDAC2 OE shows impairment in long-term contextual fear memory, the HDAC1 OE does not. Moreover, the impairment in HDAC2 OE memory is rescued with

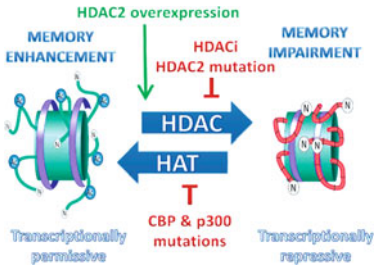


**Fig. 13.1** *Role of histone acetylation in memory consolidation*. The balance of histone acetylation is regulated by histone deacetylases (HDACs) and histone acetyl transferases (HATs). Acetylation of histones by HATs allows for a more transcriptionally permissive state of chromatin. Conversely, deacetylated or hypoacetylated histones lead to a tight bond with DNA that is transcriptionally repressive. Manipulations such as HDAC inhibitor (HDACi) treatment or HDAC2 loss-of-function mutation favor the more transcriptionally permissive hyperacetylated state and result in memory enhancement. Likewise, CBP and p300 loss-of-function mutations and dominant-negative transgene expression lead to a more transcriptionally repressive hypoacetylated state that results in memory impairment

HDAC inhibitor treatment. Histone H4K12 acetylation was significantly reduced in the HDAC2 OE but not HDAC1 OE. This residue is also acetylated in response to environmental enrichment (Fischer et al. 2007). In the *Hdac2* knockout, the authors see an enhancement in long-term contextual memory, reminiscent of HDAC inhibitor-mediated enhancement. This knockout has increased acetylation of Histone H4K5 and H4K12, as well as H2B, this last one in congruence with the HDAC inhibitor increase of acetyl H2B (Alarcón et al. 2004). In addition, chromatin immunoprecipitation with HDAC2 antibody shows association of this deacetylase with many plasticity-related genes, such as *Bdnf*, *Egr1*, and *Fos*. The study also shows that in the HDAC2 OE mice there is decreased dendritic spine density and synapse number, while the HDAC knockout mice have an increased synapse number. Overall, this study shows that HDAC2 is a target for HDAC inhibitor-mediated memory enhancement.

As we discuss later, histone acetylation is merely a component of a larger combinatorial epigenetic code, but it has proved to be quite an important component. What is more impressive about the studies on histone acetylation is that they all seem to agree that there is a balance between hyperacetylated and hypoacetylated states of histones, where the hyperacetylated state facilitates memory and synaptic plasticity, and the hypoacetylated state impairs it (Fig. 13.1).

## 13.4 Other Histone Posttranslational Modifications

Aside from acetylation, another histone modification that has been linked to memory consolidation is histone methylation. A recent study demonstrates that trimethyl-H3K4 is upregulated in hippocampus 1 h after associative learning and returns to

baseline levels at 24 h (Gupta et al. 2010). Mice deficient in Mll (H3K4 methyltransferase) have an associative memory deficit. Interestingly, an HDAC inhibitor (NaB) increased trimethyl-H3K4 and decreased dimethyl-H3K9. While H3K4 trimethylation is dependent on association of context and shock, H3K9 dimethylation occurs with context exposure alone. Mll complexed with Eed regulates neuronal plasticity, histone methylation, and HDAC recruitment (Kim et al. 2007). *Egr1* and *Bdnf* promoters show increased trimethyl-H3K4, altered DNA methylation, and methylcytosine-binding protein 2 (MeCP2) binding after contextual learning (Gupta et al. 2010). This work suggests that histone methylation results in increased DNA methylation that recruits MeCP2, paradoxically, to increase gene transcription. Interestingly, H3K9 dimethylation was also identified as the target of histone methyltransferase G9a, which has a role in cocaine-induced plasticity (Maze et al. 2010). Moreover, conditional deletion of G9a in forebrain neurons leads to reduced exploratory behavior and other behavioral abnormalities, suggesting a role for this histone methyltransferase in transcriptional homeostasis (Schaefer et al. 2009).

As alluded to previously, histone phosphorylation, another type of histone posttranslational modification, may also have a role in memory consolidation. Although it is still unclear as to whether an increase in phosphorylation is required for memory consolidation, it has been shown that histone phosphorylation increases with novel context, more so in an associative fear paradigm (Chwang et al. 2006). This increase, like the increase in histone acetylation, is dependent on the MAPK pathway. Because the same pathways regulate both acetylation and phosphorylation, and because phosphorylation increases with experience, it suggests that phosphorylation of histones, along with acetylation, regulates gene expression and therefore memory consolidation. This is addressed by a study showing that protein phosphatase 1 (PP1) is able to directly recruit epigenetic machinery, namely HDAC1 and histone demethylase JMJD2A to target genes (Koshibu et al. 2010). Inhibition of PP1 results in increased histone phosphorylation, methylation, and acetylation. Moreover, inhibition of PP1 results in enhanced long-term object recognition memory, as well as long-term spatial memory. This study suggests that histone deacetylases, demethylases, and phosphatases exist in a complex that works in concert to silence gene expression. Conversely, relief of this inhibition results in addition of these posttranslational marks to histones: acetylation, phosphorylation, and methylation.

## 13.5 Histone Variants

Gross changes in histone variant composition of the nucleosome in neurons occur mainly throughout development from Day 3 (E19) to Day 30 (P30). Nevertheless, compositions of H2A.1 and H2A.2, as well as H3.2 and H3.3, change with aging, where H2A.1 and H3.2 decrease with age, and H2A.2 and H3.3 increase with age (Pina and Suau 1987).

Local changes in histone variants are thought to be associated with active promoter and other regulatory regions (Jin et al. 2009). Specifically nucleosomes

containing variants H3.3 and H2A.Z are enriched in so-called nucleosome-free regions of active promoters, enhancers, and insulator regions. This report hypothesizes that the H3.3- and H2A.Z-containing nucleosomes are less stable than those containing the standard variants and therefore allow for dissociation from DNA, making the DNA sequence accessible to transcription factors. Although purely speculative, since there are no reported studies to confirm, it can be postulated that upon experience there is an exchange of histone variant composition. This would allow an enrichment of H3.3 and H2A.Z in active regions, leading to increased transcription and changes in gene expression that would ultimately affect behavior.

## 13.6 DNA Methylation

Many of the histone modifications described that result from learning are transient and do not explain persistent changes in gene expression, as they typically return to baseline levels before memory retrieval. Another epigenetic mark, DNA methylation, which occurs in cytosine residues, is considered more stable. DNA methylation has a demonstrated role in development and is thought to be static in nondividing, postmitotic, terminally differentiated cells. DNA methylation occurs only in cytosines that are followed by a guanine; this dinucleotide is abbreviated as CpG, where the p represents the phosphate group that covalently links the two nucleotides on the same strand. This nomenclature differentiates it from the CG base pair where each nucleotide is on opposite strands and bound primarily by noncovalent hydrogen bonds. The opposite strand of the CpG dinucleotide does, however, has a complementary CpG, which can also be methylated. CpG dinucleotides are disproportionately rare but cluster in regions in and around genes called CpG islands.

Because it was thought that DNA methyltransferases (DNMTs) do not have a role in postmitotic cells, they were not thought to be expressed in these cell populations. However, reports have shown that DNMTs are indeed highly expressed in neurons (Goto et al. 1994). Even after this report, it was unclear what the role of DNMTs was in neurons. It was speculated that these enzymes might have a role in DNA repair or neurodegeneration (Brooks et al. 1996; Endres et al. 2000). It should be noted that DNMTs are classified into two types: maintenance DNMTs, such as DNMT1, which methylate hemimethylated DNA – typically during DNA replication, and de novo DNMTs, such as DNMT3A and DNMT3B, which methylate DNA where neither strand is methylated.

A seminal study by Levenson et al. (2006) shows a role for DNMTs in plasticity-related gene regulation. This study relies on pharmacological DNMT inhibition, which decreases DNA methylation of plasticity-related gene *Reelin*, suggesting that this gene is actively methylated. Importantly, the DNMT inhibition has region-specific effects, as it increases unmethylated DNA in one CpG island of the *Bdnf* promoter I, but has no change in another CpG island of the same promoter. The study goes on to show that activation of the protein kinase C (PKC) pathway also decreases DNA methylation of the *Reelin* promoter and increases mRNA

expression of the *Dnmt3a* gene, but not *Dnmt1*. These data, together with the standing notion that DNA methylation is inhibitory, suggest that activity that leads to PKC activation leads to *Reelin* expression through decreased DNA methylation, and at the same time *Dnmt3a* expression is increased creating a negative feedback loop. However, DNMT inhibition blocks PKC-induced histone H3 acetylation, suggesting that DNA methylation recruits histone acetyltransferases, and therefore leads to transcriptional activation, whereas DNA methylation is canonically linked to transcriptional repression. This study also shows that blocking DNA methylation blocks L-LTP, which is also paradoxical, because L-LTP is associated with increased gene expression.

A follow-up study by the same research group (Miller and Sweatt 2007) looked at the role of methylation in memory formation. The mRNA expression of both *Dnmt3a* and *Dnmt3b* is increased after a contextual fear conditioning paradigm, even after subtracting context alone exposure. An interesting finding is that they are able to substantially and significantly impair long-term associative memory using DNMT inhibitors immediately after training, and that this impairment is transient. Animals are retrained without DNMT inhibitors after testing, and on the second day of testing they perform as well as controls perform on the first day of testing. This is a remarkable finding that shows the plasticity of DNA methylation, while it is widely considered to be a static mark. As stated earlier, *Reelin* gene methylation decreases with PKC activity (Levenson et al. 2006). Other genes whose methylation has been shown to be regulated are *PP1* (Miller and Sweatt 2007), *Bdnf* (Lubin et al. 2008), and *Arc* (Penner et al. 2010). *Bdnf* methylation, like *Reelin*, decreases with experience – contextual fear training, in this case – which results in higher *Bdnf* mRNA (Lubin et al. 2008). *PP1* methylation increases with experience, which results in lower *PP1* mRNA (Miller and Sweatt 2007). *Arc* is differentially methylated in promoter and intragenic regions, and this pattern is reversed from the CA1 neurons to the DG neurons of the hippocampus. Upon novel context exploration, *Arc* promoter methylation is decreased in the CA1 region, but increased in the DG region. However, in both hippocampal regions *Arc* mRNA increases with exploration (Penner et al. 2010).

As with the case of the *Hdac2* knockout recapitulating the phenotypes of HDAC pharmacological inhibition, Feng et al. (2010) show that a conditional forebrain deletion of both *Dnmt1* and *Dnmt3a* genes recapitulates the effects of pharmacological inhibitors of these enzymes in LTP, and memory. The conditional double knockout shows grossly normal synaptic properties, although it has a reduced hippocampal volume. These mutant mice show an impairment in LTP ($2 \times 100$ Hz, 20 s interval) and an enhancement in long-term depression (LTD). The mice also show deficits in spatial learning, long-term spatial memory, and in long-term contextual fear memory. Not surprisingly, overall DNA methylation levels are reduced in the conditional double knockout. Also, DNA methylation at a gene-specific level was also found to be decreased, with a concomitant increase in gene expression, as is the case for *Stat1*. Together with the previous studies looking at pharmacological inhibition of DNMTs, this study demonstrates the importance of regulated gene methylation in activity-dependent gene expression that leads to

synaptic plasticity and memory. However, the findings are contradictory to the notion that inhibition of DNA methylation, by virtue of an overall increase in transcription, would result in memory and plasticity enhancements, when in fact they are impairments.

Until recently, the identity of an active neuronal DNA demethylase was not known. *Gadd45b* is a gene that is induced in the brain in response to electroconvulsive treatment (Ploski et al. 2006), and it has had a known role in DNA damage growth arrest. Ma et al. (2009) show that neural activity induces *Gadd45b* expression in the mature hippocampus. Gadd45b was shown to be required for electroconvulsive therapy-induced demethylation of *Bdnf* promoter IX and *Fgf-1*. Furthermore, Gadd45b was found to associate with these gene regions, suggesting that it may itself act as a demethylase. Additionally, Gadd45b is required for activity-dependent adult neurogenesis, possibly through demethylation. Because no global demethylation is detected, it is postulated that Gadd45b is recruited to specific loci. Further studies on the role of Gadd45b are needed to elucidate its role in activity-dependent plasticity and memory.

Most of the studies on DNA methylation have focused on the hippocampus, and relatively transient changes in methylation status. Memory consolidation, however, may necessitate more permanent modes of memory storage. One mechanism of long-term memory storage that we discussed earlier is system consolidation, which involves the reorganization of information in brain circuitries. The most prominent example being the storage of declarative memory consolidated in the hippocampus, reorganized into the cortex. Empirical evidence of hippocampal lesions that result in anterograde amnesia (inability to form new memories) but have no effect on retrograde amnesia, supports this idea. The term for this type of memory that goes beyond days and into weeks and years is remote memory, and it is tightly associated with the cortex. Methylation, as a relatively stable epigenetic mark, is a good candidate to effect long-lasting changes in gene expression in cortical neurons. Miller et al. (2010) show that indeed gene-specific hypermethylation is induced in cortical neurons of rats, following a single, hippocampus-dependent associative learning experience. They go on to show that remote memory can be disrupted by intracortical DNMT inhibition 1 month after the learning experience.

Unlike histone acetylation, but similar to electroconvulsive treatment (ECT), DNA methylation remains somewhat of a paradox. Blocking DNMTs results in less methylation, which leads to broad gene activation and therefore would logically be associated with facilitating plasticity and memory. However, we see that DNMT inhibition, like ECT, leads to memory impairment (Fig. 13.2). With DNMT inhibition, some genes are indeed activated: *Reelin*, *Bdnf*, and *Arc*, and some are not: *PP1*. Of course the genes that are activated are positively associated with plasticity, whereas PP1 has been shown to lead to gene repression, leaving the paradox in place. One explanation for this paradox is that DNA hypomethylation is sufficient for broad transcriptional activation, in the same manner as ECT, whereas histone hyperacetylation is permissive of transcription, but not sufficient, and the refinement necessary for transcription specificity, and therefore plasticity, is determined

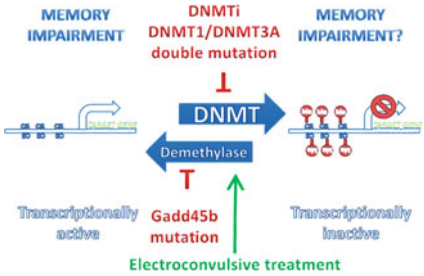


**Fig. 13.2** *Role of DNA methylation in memory consolidation*. The balance of DNA methylation is regulated by DNA methyltransferases (DNMTs) and DNA demethylases. Unmethylated or hypomethylated DNA leads to active transcription, and hypermethylated DNA leads to transcriptional inactivation. DNMT inhibitors (DNMTi) and a conditional double knockout for DNMT1 and DNMT3A result in a hypomethylated state with increased transcription, which results in memory impairment. Gadd45b mediates DNA demethylation, and a Gadd45b conditional knockout presents increased methylation and decreased gene transcription. Electroconvulsive treatment demethylates DNA through Gadd45b

by other factors. ECT has indeed been shown to actively demethylate DNA through Gadd45b, as discussed earlier (Ma et al. 2009). It is possible that some gene targets not yet studied in the context of DNA methylation may also explain this discrepancy. Also, future studies of the role of Gadd45b in demethylation may also clarify some uncertainties.

## 13.7 Combinatorial Chromatin Code

The idea of a histone code for gene expression is not a new concept, as it precedes this explosion of epigenetics in neuroscience research. What neuroscientists are considering now is how the histone code, together with DNA methylation, and histone variants form a combinatorial chromatin code that is relevant to synaptic plasticity and memory consolidation (Table 13.1).

Many studies show an interdependence of these epigenetic marks: PP1, a phosphatase that recruits a histone deacetylase and a histone demethylase (Koshibu et al. 2010), histone methylation leading to changes in DNA methylation (Gupta et al. 2010), and DNA methylation leading to histone acetylation (Levenson et al. 2006; Miller et al. 2008). What remains a question is how the transient changes in histone posttranslational modifications and DNA methylation that are observed in response to activity lead to a lasting retention of information. Nevertheless, this interplay of epigenetic marks highlights the complexity of elucidating a code. What we can recognize is that there are key players in this code – dubbed *writers* and *erasers* by Borrelli et al. (2008). The writers are those enzymes that add epigenetic marks: HATs, DNMTs, histone methyltransferases, and kinases; and the erasers take away those marks: HDACs, demethylases, and phosphatases. Moreover, these marks must be able to be *read* or decoded – task that is

**Table 13.1** Effect of pharmacological or genetic manipulations on memory consolidation

| Manipulation or mouse model | Epigenetic mechanism | Study | Effect on long-term memory |
|---|---|---|---|
| HDAC inhibition | ↑ Histone acetylation | Levenson et al. (2004) | Enhancement |
| *Creb*αδ$^{-/-}$ | ↓ Histone acetylation (through disruption of CBP interaction) | Bourtchuladze et al. (1994), Vecsey et al. (2007) | Impairment |
| *Cbp* KO | ↓ Histone acetylation | Oike et al. (1999), Bourtchuladze et al. (2003) | Impairment |
| *Cbp*$^{+/-}$ | ↓ Histone acetylation | Alarcón et al. (2004) | Impairment |
| *Cbp{HAT-}* | ↓ Histone acetylation | Korzus et al. (2004) | Impairment |
| *CbpΔ1* | ↓ Histone acetylation | Wood et al. (2005) | Impairment |
| *Cbp*$^{kix/kix}$ | ↓ Histone acetylation | Wood et al. (2006) | Impairment |
| *p300 Δ1* | ↓ Histone acetylation | Oliveira et al. (2007) | Impairment |
| *p300*$^{+/-}$ | ↓ Histone acetylation | Viosca et al. (2010) | No change (Impairment in relearning) |
| *Pcaf* KO | ↓ Histone acetylation | Duclot et al. (2010) | Impairment (acquisition) |
| *Hdac2* KO | ↑ Histone acetylation | Guan et al. (2009) | Enhancement |
| *Hdac1* OE | ↓ Histone acetylation | Guan et al. (2009) | No change |
| *Hdac2* OE | ↓ Histone acetylation | Guan et al. (2009) | Impairment |
| *Mll* KO | ↓ Histone methylation | Gupta et al. (2010) | Impairment |
| *NIPP1* | ↑ Histone phosphorylation | Koshibu et al. (2010) | Enhancement |
| DNMT inhibition | ↓ DNA methylation | Levenson et al. (2006), Miller and Sweatt (2007) | Impairment |
| *Dnmt1*/*Dnmt3a* KO | ↓ DNA methylation | Feng et al. (2010) | Impairment |
| ECT | ↓ DNA methylation (through Gadd45b) | Duncan (1949), Ma et al. (2009) | Impairment |

Several mouse models and other manipulations in rodents have consistently demonstrated that epigenetic mechanisms are able to modulate memory consolidation. The table summarizes each manipulation, with its effect on epigenetic state, and its ultimate memory phenotype

performed through protein domains such as bromo and chromo domains that recognize these marks. The interplay of these marks comes in that many of the domains that *read* them are in writers and erasers themselves. HATs have bromo domains that recognize histone acetylation and therefore propagate this mark. HDACs are recruited by MeCP2, which recognizes methylated DNA. Ultimately, much of this code will be deciphered through high throughput proteomics and methyl-cytosine sequencing.

## 13.8 Higher Order Chromatin Structure

Recent evidence shows that CBP recruitment is not only in gene promoter regions, but also in enhancer regions, which play a more central role in multiple gene regulation (Kim et al. 2010). The presence of CBP in these regions,

outside of promoters, prompts the question of the role of these enhancers in memory consolidation. When we think of gene regulation, our vision is somewhat limited by the depictions that we use. The nucleus is a very crowded space, and subnuclear chromosomal arrangements are important for the regulation of gene expression. Enhancer and insulator regions have special importance as they can modulate expression in this crowded space, with enhancers aiding in gene activation by bringing elements together, and insulators aid in separating elements and avoiding spurious gene activation. Chromosomal organizers, such as SATB1 (Cai et al. 2006), may well be the target of regulation in response to neuronal activity. DNA is held in large loops thanks to these organizers, and these loops have torsional strains if they are actively transcribed. So another level of regulation to consider is the role of topoisomerases, and alternative DNA structures, in gene regulation. Future studies will elucidate the role of these higher order structures in activity-dependent regulation of transcription.

## 13.9 Concluding Remarks

As stated in a review by Roth and Sweatt (2009), the field of epigenetics in neuroscience has created a paradigm shift in the locus of plasticity. Much attention had been given to the synapse and to synapse-specific regulation. Now we have compelling evidence for a role of the nucleus in modulating plasticity. These recent findings on regulation of plasticity at the level of the nucleus do not allow us to discard the concept of synapse-specific regulation. There are certainly different tiers of regulation where one nucleus with one genome is able to somehow orchestrate synapse-specific plastic changes. Epigenetic marks are able to explain persistent changes in gene expression, but they do not explain how those changes translate to the synapse. However, examples like BDNF expression give us an idea of how this can occur. *Bdnf* transcription is regulated by DNA methylation, histone methylation, and histone acetylation (Lubin et al. 2008; Gupta et al. 2010; He et al. 2010), and its mRNA once transcribed has another level of regulation: dendritic trafficking (reviewed in Lu 2003; Tongiorgi 2008). This is regulated by mRNA-binding proteins that can target the message to specific synapses and also by regulating local translation at those synapses. Lastly, there is a vast virtually uncharacterized collection of noncoding RNAs whose gene regulation would certainly also depend on the state of chromatin. These RNAs can modulate mRNA permanence and therefore provide another means for regulation that has not been fully incorporated into the big picture (Mercer et al. 2008). Overall, the regulation of the state of chromatin as part of neuronal plasticity is a burgeoning field, and specifically for memory, it has had a profound impact in our understanding of long-term information storage.

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# Chapter 14
# Epigenetic Mechanisms in Memory Formation

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**Abstract** Formation of memories of events in our lives is one of the principal functions of the brain. We make particularly strong memories of events with an emotional impact. Glucocorticoid hormones, secreted in response to the stressful event, have been identified as playing an important role in the acquisition and consolidation of such memories. In recent years, significant advances have been made in the identification of the signaling and epigenomic mechanisms in the hippocampus underlying memory formation. Evidence has been accumulating for a principal role of the NMDA–ERK MAPK signaling pathway and its downstream effector molecules MSK1 and Elk-1. Activation of this signaling cascade results in the phosphorylation, acetylation, and possibly methylation of histone molecules within the chromatin structure and in the induction of immediate-early (e.g., c-Fos) and many other genes required for the molecular and cellular adaptation of the affected neurons. Glucocorticoid hormones via the glucocorticoid receptor (GR) enhance memory formation through facilitation of ERK MAPK signaling to the chromatin leading to the enhancement of epigenomic mechanisms and cognitive performance. Thus, formation of strong memories of emotional events involves an interaction between the GR and the NMDA/ERK/MSK1 and Elk-1 signaling pathways resulting in optimization of epigenomic changes in hippocampal neurons to allow the induction of required neuroplasticity changes.

**Keywords** Acetylation · Behavior · c-fos · Chromatin · Cognition · Elk-1 · ERK · Glucocorticoid · Histone · Learning and memory · MAPK · MSK · NMDA · Phosphorylation · Resilience · Stress

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A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1_14,
© Springer-Verlag Berlin Heidelberg 2011

One of the principal functions of the brain is the formation of memories of experienced events. Particularly, memories of emotional events are strong and sometimes lasting for life. Possibly, importance is given to such memories because they help the organism to adapt and respond better if similar events would reoccur in the future. Therefore, mostly memories are beneficial for survival, health, and well-being. However, disruptions in this cognitive process may play a role in stress-related psychiatric disorders such as major depression and posttraumatic stress disorder (PTSD).

The neurobiological mechanisms underlying learning and memory have been mostly studied in rodents such as rats and mice. Well-known learning and memory paradigms include the Morris water maze, the radial maze and the forced swim test. In these tests the animal learns to choose the most appropriate behavioral response to enhance the chance of survival [finding the platform in the Morris water maze (Morris 1984); conservation of energy by floating in the forced swim test (Bilang-Bleuel et al. 2005; De Pablo et al. 1989; Korte 2001; West 1990)] and reward (food in the radial maze)]. Clearly, many of these behavioral tests involve aversive and anxiogenic conditions (e.g., novelty, fear, and sleep deprivation), and stress hormones such as glucocorticoid hormones (corticosterone in rats and mice) are secreted during the learning sessions (Bilang-Bleuel et al. 2005; Droste et al. 2008; Peñalva et al. 2003). Thus, secretion of glucocorticoids and other stress hormones (e.g., adrenalin and noradrenalin) is inherent to most behavioral tests in rodents.

Notably, the stress response and the learning and memory processes have in the past been separately described, but it may be argued that they actually constitute highly integrated biological mechanisms. It has been shown that the released glucocorticoid hormones strongly facilitate the formation of memories of the experienced event. The memory-enhancing effects of glucocorticoid hormones have been described in many behavioral tests such as Morris water maze behavior (Oitzl and De Kloet 1992), fear conditioning (Roozendaal et al. 2006), the forced swim test (Bilang-Bleuel et al. 2005; De Kloet et al. 1988; Korte 2001; Korte et al. 1996) and others (Beylin and Shors 2003; Smeets et al. 2009). Although these facilitatory actions of glucocorticoids have been known for many years, the question of how glucocorticoids act on learning and memory processes has remained unanswered. However, recent findings based on signaling and epigenomic studies have substantially increased our insight into the underlying mechanisms of glucocorticoid action on learning and memory.

## 14.1 Gene Transcription-Related Epigenetic Mechanisms

It is now well established that gene transcription is largely controlled by epigenetic mechanisms at the chromatin level. Epigenetic mechanisms affecting the chromatin structure and function include the covalent modifications of histone molecules and the methylation of DNA. Here we will focus on the role of histone modifications. Histone proteins such as histone H3 have evolutionary highly conserved N-terminal tails which stand out from the nucleosome and can be subjected to posttranslational modifications such as acetylation, phosphorylation, methylation and others (Strahl and Allis 2000).

These histone modifications, and more importantly the combination of various histone modifications, determine the functional state of the chromatin. The acetylation of Lysine amino acids in histone H3 and H4 is seen in open, transcriptionally active chromatin (Strahl and Allis 2000). Some immediate-early genes such as *c-fos* and *c-jun* (Clayton et al. 2000), and other genes [e.g., matrix metalloprotease-1 (MMP-1) (Martens et al. 2003)] require the phosphorylation of Serine-10 (S10p) combined with the acetylation of Lysine-14 (K14ac) for induction of gene expression. The specific combination of histone H3 modification may be required for the recruitment of specific nuclear factors to the chromatin to allow induction of transcription. The methylation of histone H3 tails is associated with transcriptional activation as well as gene silencing. Methylation of the H3K4 mark results in gene activation whereas H3K9 and H3K27 methylation leads to gene silencing (Akbarian and Huang 2008). The combination of the H3K9 methylation and H3S10 phosphorylation marks is associated with gene silencing (Sabbattini et al. 2007). As the combinatorial H3S10p-K14ac marks are thought to play a role in the local opening of condensed, inactive chromatin these histone modifications may be crucial for the transcriptional activation of dormant genes (Cheung et al. 2000; Clayton et al. 2000). However, whether the H3S10p-K14ac marks are required for any gene located in condensed, heterochromatin seems questionable. Even the necessity of these histone marks for the induction of *c-fos* may depend on the cell type or tissue under investigation (see below).

## 14.2 Psychologically Salient Events Evoke Gene Transcription-Related Histone Modifications in the Brain

It was a serendipitous finding around the turn of the millennium when we found the H3S10p mark in neurons of the rat and mouse brain (Bilang-Bleuel et al. 2000). Using an antibody against H3S10p (also recognizing H3S10p acetylated at Lys14, i.e. H3S10p-K14ac [(Chandramohan et al. 2007), Chandramohan and Reul, unpublished observations] neurons were found showing a speckled nuclear immunostaining pattern. These neurons were mainly found in the dentate gyrus of the hippocampus and there were only few neurons scattered in the amygdala, neocortex and striatum (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007). Our studies indicated that histone H3 if phosphorylated at Ser10 will be acetylated at Lys14, thus forming H3S10p-K14ac (Chandramohan et al. 2007). As we were interested in how animals adapt to and learn from psychologically stressful events we studied whether such challenges would affect the number of neurons expressing H3S10p-K14ac. Challenging rats or mice with forced swimming, a predator or a novel environment, led to a substantial rise in the number of H3S10p-K14ac-positive neurons specifically in the dentate gyrus (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007, 2008). The increase peaked at 1–2 h after the challenge and returned to baseline levels after approximately 4 h (Chandramohan et al. 2007, 2008). Thus, the response was relatively fast and transient showing that epigenetic changes underlying

gene expression can be highly dynamic. Morris water maze learning [Chandramohan and Reul, unpublished observations; (Chwang et al. 2007)] and fear conditioning [Chandramohan Y, Sacchetti B, Strata P, and Reul JMHM, unpublished observations; (Chwang et al. 2007)] also resulted in increases in the number of H3S10p-K14ac-expressing neurons in the dentate gyrus. The question arose why the combinatorial H3S10p-K14ac mark in dentate neurons responded similarly to such different stimuli? The response pattern of H3S10p-K14ac-positive neurons in the dentate gyrus indeed does not give clues about the challenge the animal has undergone; see for instance references (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007, 2008). These findings nonetheless correspond with the role of the dentate gyrus in learning and memory processes.

## 14.3 Sparse Epigenetic Responses in the Dentate Gyrus

The dentate gyrus represents a part of the hippocampus which is one of the principal structures of the limbic system. It plays a major role in learning and memory processes. As the main neuroanatomical gate of the hippocampus, the dentate gyrus receives inputs from the entorhinal cortex, the principal neocortical region that feeds integrated sensory and other information into the limbic system (Witter 2007). After processing, the dentate neurons pass this information on to pyramidal neurons in the other hippocampal cell fields (mainly CA3) where the information is integrated with other, stimulus-specific information for further processing to yield appropriate physiological and behavioral responses and, ultimately, memory formation of the event (Rolls and Kesner 2006; Treves and Rolls 1994). Thus, when animals are challenged, as a result of the sensory information flow, granule neurons in the dentate gyrus are activated. GABAergic interneurons exert a high tonic inhibitory control on dentate granule neurons and therefore only relatively few granule cells (<5%) become activated. Such sparse activation occurs irrespective of the stimulus (e.g., novelty, forced swimming, Morris water maze learning; in contrast, strong depolarizing agents, such as kainate, or electroconvulsive shocks evoke an all-out activation) (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007, 2008; Chawla et al. 2005; Rolls and Kesner 2006). Indeed, recently we showed that GABA is an important modulator of baseline and novelty-evoked H3S10p-K14ac and c-Fos in dentate neurons (Papadopoulos et al. 2008) (see below). Thus, the neuronal activation pattern in the dentate gyrus seems to be a reflection of the degree to which prominence is given to afferent sensory stimuli. It appears that the enhanced H3S10p-K14ac expression in dentate granule neurons after such stimuli is part of the sparse activation response of this hippocampal region.

Despite the abundance of evidence favoring a role of the H3S10p-K14ac marks in gene activation, until now surprisingly few genes have been identified whose expression depends on these epigenetic marks. Mahadevan et al. demonstrated in in vitro cell culture experiments that phosphoacetylated histone H3 is associated with the induction of the immediate-early genes *c-fos* and *c-jun* (Clayton et al. 2000). Later studies of

Martens and colleagues showed that H3S10p is involved in MMP-1 induction (Martens et al. 2003). We showed for the first time in vivo that the H3S10p-K14ac marks in rat and mouse dentate granule neurons are associated with c-Fos induction (Chandramohan et al. 2007, 2008; Gutierrez-Mecinas et al. 2009). A first indication was founded on the strictly parallel changes in H3S10p-K14ac-positive and c-Fos-positive neurons after various experimental manipulations and the colocalization of the epigenetic mark and gene product in the same dentate neurons based on immunofluorescence analyses (Chandramohan et al. 2007, 2008). Recently, using chromatin immunoprecipitation (ChIP) and qPCR we showed that forced swimming indeed evoked the combinatorial histone marks in the *c-fos* promoter region in dentate neurons (Gutierrez-Mecinas et al. 2009). Moreover, forced swimming also resulted in histone H4 hyperacetylation, but not H3 hyperacetylation, in this promoter (Gutierrez-Mecinas et al. 2009). A similar pattern of histone modification marks has been reported for the hippocampal *c-fos* promoter after electroconvulsive shock treatment which is known to elicit a full-blown c-Fos induction in the hippocampus including the dentate gyrus (Tsankova et al. 2004). In contrast, the neocortex known as well to induce c-Fos after forced swimming (Bilang-Bleuel et al. 2002) presented a different pattern of epigenetic marks at the c-fos promoter. ChIP revealed hyperacetylation of H4 in the c-fos promoter after forced swimming but no changes in H3S10p-K14ac and acetylated H3 (Gutierrez-Mecinas et al. 2009). These observations confirm earlier findings that the rise in H3S10p-K14ac observed after psychological challenges such as forced swimming and novelty is exclusively occurring in the dentate gyrus and is not seen elsewhere in the brain (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007, 2008). Thus, in different neuronal populations in the brain, expression of the same gene may be driven by epigenetically diverse mechanisms. Furthermore, in different neurons, epigenetic mechanisms controlling expression of the gene may be steered by distinct signaling pathways.

## 14.4 Signaling to the Chromatin Involves Integration of Extracellular and Intracellular Pathways

### *14.4.1 Involvement of the NMDA Receptor and Glucocorticoid Receptor*

For survival, an organism needs to adapt to and learn from challenges imposed by its environment. In recent years a picture is emerging that cognitive processing of environmental challenges involves changes in epigenetic mechanisms and gene expression profiles in multiple populations of neurons. The environment impacts on these intranuclear events through a coordinated activation of extracellular (e.g., hormones and neurotransmitters) and intracellular signaling pathways. It is however still early days with regard to our understanding how epigenetic mechanisms are steered by signaling molecules. Recently we reported that the neurotransmitter glutamate and the glucocorticoid hormone corticosterone (acting through the

*N*-methyl-D-aspartate (NMDA) receptor and the glucocorticoid receptor (GR), respectively) are both required for the forced swimming- and novelty-induced histone H3 phosphoacetylation and c-Fos induction in dentate neurons (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007, 2008). The requirement for activation of both pathways was additionally substantiated by the finding that the sole injection of rats with a GR-occupying dose of corticosterone was ineffective (Chandramohan et al. 2007). Glucocorticoid hormone action via the mineralocorticoid receptor and the gaseous messenger nitric oxide is not involved (Chandramohan et al. 2007, 2008), suggesting specificity in participating mediators.

## 14.5 GABAergic Control of Dentate Gyrus Epigenomic Responses

On the basis of electrophysiological and computational studies, it is thought that the encoding of sensory information within the dentate gyrus is conducted orthogonally by sparsely distributed granule neurons (Rolls and Kesner 2006). The sparse neuronal activation pattern is required for appropriate information processing (Leutgeb et al. 2007; Rolls and Kesner 2006) and involves an important role of the strong tonic inhibitory control exerted by local GABAergic interneurons (Rolls and Kesner 2006; Treves and Rolls 1994). Thus, granule neurons are only excited if the stimulus is strong enough to overcome the GABAergic inhibitory tone. The excitation is brought about by glutamate acting via NMDA receptors (Collingridge and Singer 1990; McHugh et al. 2007; Richter-Levin et al. 1995; Treves and Rolls 1994).

Aspsychological challenges evoke a sparse pattern of H3S10p-K14ac and c-Fos in dentate granule neurons, we hypothesized that GABA may be an important modulator of such epigenomic responses. We found that pretreatment of rats with the benzo Lorazepam, an indirect GABA-A receptor agonist, dose-dependently blocked the effect of a novel cage challenge on histone H3 phosphoacetylation and c-Fos induction in dentate neurons (Papadopoulos et al. 2008). This inhibition was accomplished at a dose of the benzo that was found to be anxiolytic but not sedative. Conversely, the partial inverse GABA-A agonist FG-7142, a drug that is known to attenuate the GABAergic inhibition of dentate granule neurons, profoundly enhanced baseline levels as well as novelty-induced increases in the number of H3S10p-K14ac- and c-Fos-positive dentate neurons (Papadopoulos et al. 2008). Corresponding with previous reports, after FG-7142 the rats showed anxiety-like behavior and hypervigilance in the novel cage. Furthermore, the FG-7142-evoked enhancements in epigenomic changes were found to be completely blocked by the NMDA receptor antagonist MK-801, which underscores the critical importance of this glutamate receptor in dentate granule neuron activation (Papadopoulos et al. 2008).

These observations confirm that GABA functions as a major controller in the dentate gyrus. This function precipitates at least in part through its modulation of epigenomic responses in the granule neurons. Thus, regulators of GABA activity in

the dentate gyrus may modulate the extent to which salience is given to incoming sensory information.

## 14.6 ERK–MSK Signaling Drives the Histone H3S10 Phosphorylation Mark

In general, relatively little is known about the signaling mechanisms involved in the activation or inhibition of histone modifying enzymes. The signaling cascade mediating the phosphorylation and acetylation of histone H3 has, however, been rather well established. Pharmacological and mutant mouse studies have indicated the involvement of the extracellular signal-regulated kinases ERK1/2 and the mitogen- and stress-activated kinases MSK1/2 (Chandramohan et al. 2008). ERK1/2 has been shown to be activated by phosphorylation via the mitogen-activated protein kinase (MAPK) pathway after NMDA receptor stimulation (Sweatt 2004). pERK1/2 (as well as p38MAPK) can phosphorylate MSK1 at Ser-360, Thr-581, and Thr-700 after which MSK1 will autophosphorylate itself at multiple sites resulting in full catalytic – H3S10 kinase – activity (Arthur 2008; Hauge and Frodin 2006). Dentate gyrus neurons are known to express NMDA receptors, ERK1/2 and MSK1, but it was unclear whether pERK1/2, pMSK1, H3S10p-K14ac, and c-Fos would actually come to expression in the same neurons after a psychological challenge. The often practiced Western blot analysis would, of course, not provide any clues in this regard. Recently, in a series of immunofluorescence studies we could demonstrate the colocalization of pERK and pMSK1 in dentate granule neurons (Gutierrez-Mecinas et al. 2009). Moreover, we could show that pERK1/2, pMSK1, and H3S10p-K14ac are expressed in the same dentate neurons after a forced swim challenge providing clear evidence that H3S10 phosphorylation is the result of NMDA/ERK1/2/MSK1 signaling in these neurons (Gutierrez-Mecinas et al. 2009) (Fig. 14.1). In addition, neither expression of another MSK kinase, i.e., phospho-p38MAPK, nor expression of the MSK-related kinase, pRSK1/2, was found in dentate granule neurons (Gutierrez-Mecinas et al. 2009). Thus, there is specificity in the signaling mechanisms recruited to convey environmental challenges to the neuronal chromatin.

## 14.7 Establishment of the Combinatorial H3S10p-K14ac Marks: K14 Acetylation of H3S10p

Some time ago, we proposed on the basis of the following observations that pCREB-CBP may be responsible for the K14 acetylation in H3S10p (Chandramohan et al. 2008; Reul and Chandramohan 2007; Reul et al. 2009) (1) psychological challenges such as forced swimming result in a strongly increased phosphorylation of the transcription factor CREB in dentate gyrus neurons (Bilang-Bleuel et al. 2002); (2) MSK is, in addition to a H3S10 kinase, also a CREB kinase (Arthur and Cohen 2000); (3) the

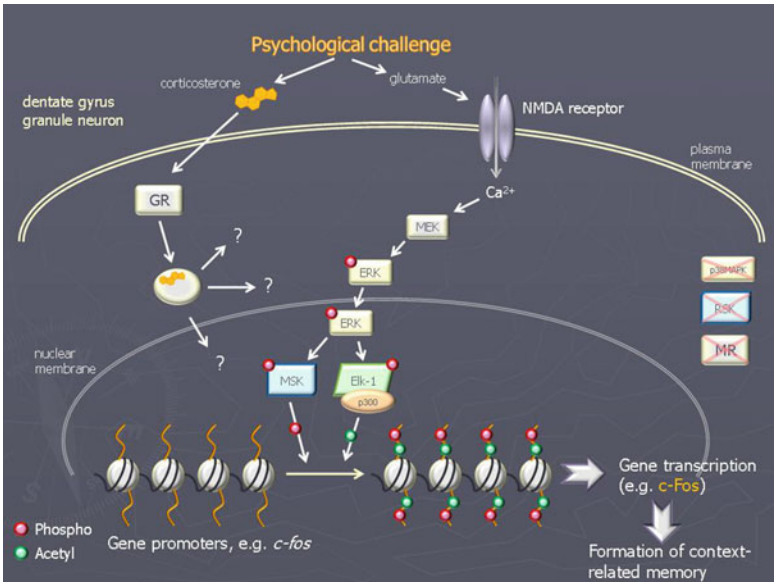


**Fig. 14.1** A psychological challenge impacts on epigenomic mechanisms and memory formation through convergent activation of the GR- and NMDA–ERK-driven MSK1 and Elk-1 signaling pathways. Activation of these pathways leads to S10-phosphorylation and K14-acetylation of histone H3, hyperacetylation of histone H4, transcriptional induction of c-Fos (and other genes) in a distinct population of mature dentate granule neurons (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007, 2008; Gutierrez-Mecinas et al. 2009), and the encoding of contextual memory of the endured event. Our research has shown that factors such as the MSK kinase p38MAPK, the MSK-related kinase RSK, and the glucocorticoid-binding mineralocorticoid receptor (MR) are not involved in the observed epigenetic, gene expression, and cognitive phenomena

*c-fos* gene promoter contains a CRE site; and (4) pCREB is able to recruit CREB-binding protein (CBP/p300, proteins with histone acetyl transferase (HAT) activity) to the promoter (Schiltz et al. 1999). However, after forced swimming phosphorylation of CREB takes place in virtually all dentate gyrus neurons (Bilang-Bleuel et al. 2002), which is in stark contrast to the sparse H3S10p-K14ac and c-Fos induction. Therefore, although a role of pCREB/CBP cannot be entirely excluded, pCREB may be playing a more general, neuroprotective role (Papadia et al. 2005) in the dentate gyrus after a psychological challenge. Recently, we discovered that challenges such as forced swimming and novelty result in the phosphorylation of the E twenty-six (ETS)-domain protein Elk-1 [Ets-like protein-1 (Sharrocks 2001; Shaw and Saxton 2003; Yordy and Muise-Helmericks 2000)] specifically in pERK1/2/pMSK1/H3S10p-K14ac/c-Fos-positive neurons of the dentate gyrus (Gutierrez-Mecinas et al. 2009). According to in vitro studies Elk-1 can be activated through ERK MAPK signaling (Yang et al. 2003a, b). Moreover, pElk-1 bound to the Elk-1-binding site within the serum response element (SRE) of the *c-fos* promoter recruits HATs like p300 to the promoter that subsequently acetylate histone molecules in adjacent nucleosomes (Li et al. 2003a; O'Donnell et al. 2008). Thus, ERK1/2-driven Elk-1 phosphorylation in dentate

neurons may drive the acetylation of H3S10p (and H4) in the *c-fos* promoter (Fig. 14.1). This notion is supported by our recent immunofluorescence data showing the colocalization of pElk-1 with pERK1/2, pMSK1, H3S10p-K14ac, and c-Fos.

## 14.8 Glucocorticoid Receptor Involvement in Histone H3 Phosphoacetylation and c-fos Gene Expression in Dentate Gyrus Neurons

In a series of studies, we demonstrated that the establishment of the combinatorial H3S10p-K14ac marks and consequent c-Fos induction in dentate gyrus neurons requires in addition to signaling through the NMDA/ERK1/2/MSK1 and Elk-1 pathway also a GR-mediated action (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007, 2008). Presently, however, it is unclear at which level(s) the distinct signaling pathways are interacting (Fig. 14.1). Classically, GRs act as ligand-dependent transcription factors altering gene expression through interaction with glucocorticoid responsive elements (GREs) in promoter regions of glucocorticoid responsive genes. As challenge-induced H3 phosphoacetylation is rather quick (significant increases within 15 min), a role of a glucocorticoid-induced gene product is unlikely. However, GRs may also be acting through nongenomic mechanisms. GRs can interact with different signaling pathways among which the MAPK ERK signaling pathway (Revest et al. 2005). Similar to the demonstrated interaction of the progesterone receptor (PR) with ERK1/2 to produce MSK activation (Vicent et al. 2006), we propose, based on the strong similarity between the GR and the PR, that the GR may be required for the full activation of MSK1. Alternatively, GRs have been shown to recruit chromatin-remodeling proteins such as ATP-dependent chromatin-remodeling complexes and histone modifying enzymes such as HATs (e.g., pCAF), thereby promoting chromatin decondensation, histone acetylation, and transcriptional activation (Hebbar and Archer 2003; Kinyamu and Archer 2004; Li et al. 2003b). Thus, GRs interact with signaling pathways and the chromatin in a highly complex manner and clearly more research is required. Yet, it appears that activated GRs are of crucial importance in the facilitation of NMDA/ERK/MSK and Elk-1 signaling to the chromatin.

## 14.9 Importance of H3S10p-K14ac-Associated Gene Expression in Dentate Gyrus Granule Neurons in Hippocampus-Related Memory Formation

Using well-characterized primary antibodies and immunofluorescence analysis it is now possible to demonstrate epigenetic mechanisms linked to specific gene expression events (e.g., c-Fos induction) within single cells in the brain. Moreover, intracellular

molecules (e.g., pERK1/2, pMSK1, and pElk-1) can be traced signaling to the chromatin, thereby affecting gene expression. Over the last decade an impressive collection of data have been accumulating that strongly support a role of the combinatorial H3S10p-K14ac epigenetic marks and associated gene expression in hippocampus-associated learning and memory processes. Behavioral tests for hippocampus-associated memory formation include the forced swim test, Morris water maze learning, and contextual fear conditioning. In view of its role in sensory information processing and encoding, the dentate gyrus is critically involved in memory formation in these tests (Rolls and Kesner 2006; Treves and Rolls 1994). With regard to the formation of memories of a forced swim experience [for details on the forced swim test, see (Chandramohan et al. 2008)], a strict requirement was the generation of H3S10p-K14ac and c-Fos in dentate granule neurons after the initial test, thus during the acquisition and consolidation phase of memory formation. If the generation of H3S10p-K14ac and c-Fos in these neurons was disrupted due to NMDA receptor or GR blockade, MEK (MAPK kinase) inhibition (thereby preventing of ERK activation), or MSK1/2 gene knockout, formation of memory of the event was greatly impaired (Chandramohan et al. 2007, 2008; Reul and Chandramohan 2007; Reul et al. 2009). Correspondingly, antagonism of the mineralocorticoid receptor [MR; another glucocorticoid-binding receptor in the brain (Reul and De Kloet 1985)] neither affected memory formation of forced swim experience [as shown before (Veldhuis et al. 1985)] nor H3S10p-K14ac and c-Fos in the dentate gyrus (Chandramohan et al. 2008).

Recently, first epigenetic data have been collected in exercising animals. Long-term voluntary exercise has been shown to result in enhanced cognition, reduced anxiety and impulsiveness, and distinct changes in glucocorticoid hormone responses (Binder et al. 2004; Droste et al. 2007, 2009; van Praag et al. 1999). Indeed, exercising rats show enhanced H3S10p-K14ac and c-Fos responses in their dentate gyrus to forced swimming (and novelty) and make stronger memories of the experienced event (i.e., forced swimming) than the sedentary controls (Collins et al. 2009). Exercised rats show increased hippocampal GR expression (Droste et al. 2007, 2009), which may have led to an enhanced facilitation of ERK1/2/MSK1 and Elk-1 signaling and thus to an enhanced epigenomic impact. This hypothesis is currently under investigation.

There is preliminary evidence that Morris water maze learning involves H3S10p-K14ac and c-Fos induction in dentate neurons (Chandramohan and Reul, unpublished observations). Indeed, using whole hippocampus extracts and Western analysis, Chwang et al. (2007) observed a role of H3S10p in memory formation in the Morris water maze and contextual fear conditioning requiring signaling through ERK1/2 and MSK1. Presently, a role of histone H3 and H4 methylation is emerging. The role of histone methylation is complex as lysine residues can carry up to three methyl groups and the degree of methylation can have implications for chromatin structure and transcriptional activity. Mono-, di-, and trimethyl H3K4 are all associated with transcriptional activation (Akbarian and Huang 2008). However, the monomethylation marks of K9 and K27 of H3 and K20 of H4 are linked with gene activation, whereas the di- and trimethylation marks of these residues are associated with gene repression (Akbarian and Huang 2008). It should be emphasized that these findings are based on in vitro studies and until now only few reports exist on histone

methylation-associated changes in neuronal gene expression in vivo [e.g., (Huang et al. 2007; Schaefer et al. 2009)]. We found preliminary evidence for an increased H3K4 methylation of the *c-fos* promoter in the hippocampus after forced swimming (Hesketh and Reul, unpublished observations). McEwen and colleagues recently reported changes in overall levels of H3K4, H3K9, and H3K27 methylation in the brain of acutely and chronically (restraint) stressed rats (Hunter et al. 2009). Conditional mutagenesis of H3K9 methyltransferase complex GLP/G9a in mice resulted in altered exploratory, locomotor, and cognitive behaviors (Schaefer et al. 2009). Gupta et al. demonstrated that H3K4 methylation is involved in the establishment of contextual fear memories (Gupta et al. 2010). Thus, we are only beginning to understand how major life events impact on epigenomic mechanisms in neurons participating in the formation of memories of such events.

## 14.10 Concluding Remarks

There are now clear indications that epigenetic mechanisms controlling gene transcription play a key role in learning and memory processes. Valuable information has also been gathered about how these epigenetic mechanisms are controlled by intracellular and extracellular signaling pathways. In this chapter, we described the role of NMDA/ERK1/2/MSK1 and Elk-1 signaling, GR activation, and GABA-A-mediated control on histone H3 S10-phosphorylation and K14-acetylation, hyperacetylation of H4, and subsequently induction of c-Fos in dentate granule neurons in response to an emotional or otherwise psychologically stressful event. These epigenomic mechanisms appear to be of critical importance for storing memories of such events. Histone methylation and demethylation events may be playing an important role as well. How glucocorticoid hormones strengthen memory formation is still elusive, but recent evidence suggests that glucocorticoids, in part, may act through facilitation of ERK MAPK signaling in dentate neurons leading to enhanced epigenomic responses in these neurons. Elucidation of these epigenomic processes may be key to resolve psychiatric illnesses such as major depression and anxiety-related disorders [e.g., PTSD (Reul and Nutt 2008)] in a not too distant future.

**Acknowledgment** Our work described in this paper is supported by the Biotechnology and Biological Sciences Research Council (BBSRC; Grant Reference Number BB/F000510/1) of the United Kingdom.

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# Glossary

**5-Aza-2-deoxy-cytidine (Decitabin)** A cytosine in which the 5 carbon of the cytosine ring has been replaced with nitrogen. Decitabone is exclusively incorporated in DNA inhibiting mammalian *DNA methyltransferases*.

**5-Azacytidine (AZA)** A cytidine RNA analog in which the 5 carbon of the cytosine ring has been replaced with nitrogen. 5-Azacytidine can be incorporated in RNA and after metabolic activation also in DNA, where it functions as an inhibitor of mammalian *DNA methyltransferases*.

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

**Adrenocorticotropin hormone (ACTH)** A polypeptide tropic hormone secreted by the anterior pituitary gland in response to biological stress.

**Agouti gene** The murine agouti gene (A) controls fur color 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 and the other from the father. The DNA sequence of each of these copies may be different because of genetic polymorphisms.

**Angelman syndrome (AS)** A rare pediatric diseases caused by chromosomal aberrations or epigenetic inactivation of genes on the maternal chromosome 15.

**Anxiety disorders** Disorders with different forms of abnormal and pathological fear and anxiety.

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

**ATRX** Alpha-thalassemia/mental retardation syndrome X-linked (ATRX) is a protein that belongs to the switch/sucrose nonfermentable (SWI/SNF) family of chromatin remodeling proteins, which facilitate gene expression by allowing

A. Petronis and J. Mill (eds.), *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, DOI 10.1007/978-3-642-17426-1,
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transcription factors to gain access to their targets in chromatin. Mutations in the ATRX gene alter DNA methylation and have been associated with an X-linked mental retardation syndrome that is often accompanied by alpha-thalassemia (ATRX) syndrome.

**Autism** A neuropsychiatric disorder characterized by impaired social interaction and communication, and by restricted and repetitive behavior.

**Bipolar disorder (BPD)** A psychiatric disease defined by the presence of one or more episodes of abnormally elevated energy levels, cognition, and mood with or without one or more depressive episodes.

**Bisulfite genomic sequencing** A procedure in which sodium 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 reveal 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.

**Brain-derived neurotrophic factor (BDNF)** A protein which acts on certain neurons of the central and peripheral nervous system, supporting the survival of neurons and encouraging the growth and differentiation of new neurons and synapses.

**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.

**CBP** CREB-binding protein involved in transcriptional regulation often associating with histone acetyltransferases such as p300.

**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 organization of *chromatin* – DNA and the histone proteins – in the nucleus.

**Cellular Memory (epigenetic)** Specific active and repressive organizations 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.

**Cerebellum** Region of the brain that plays a role in motor control, as well as language, attention, and some elements of emotion.

**Cerebral cortex** A sheet of neural tissue covering the mammalian cerebrum.

**ChIP** See *chromatin immunoprecipitation*.

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

**ChIP-seq** Sequencing of the totality of DNA fragments obtained by ChIP using next-generation sequencing to quantify patterns of enrichment across the genome.

**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 immunoprecipitation (ChIP)** This is a method for examining protein–DNA interactions occurring in the cell. DNA-binding proteins are cross-linked to the DNA and enriched using antibodies with specific affinity to 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 analyzed by qPCR, microarray (ChIP-chip), or next-generation sequencing (ChIP-seq).

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

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

**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 approximately 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 organized (e.g., 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*.

**Corticotropin releasing hormone (CRH)** A polypeptide hormone and neurotransmitter involved in the stress response.

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

**CREB** cAMP response element-binding protein, a transcriptional activator for many immediate early 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).

**Dentate gyrus** Part of the hippocampal formation believed to contribute to memory formation and other brain functions.

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

**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.

**Dopamine** A catecholamine neurotransmitter that has an important role in cognitive function, voluntary movement, reward, motivation, and prolactin production.

**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.

**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-characterized endocrine disruptor is *Bisphenol-A*, a chemical used for the productions of certain plastics.

**Enhancer** A small, specialized sequence of DNA which, when recognized 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 fertilization through a sequence of steps leading to a gradual increase in complexity through differentiation of cells and formation of organs.

**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 inheritance, 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*.

**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.

**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.

**Escape of X-inactivation** Regions and genes on the X-chromosomes which are not affected by the dosage compensation/X-inactivation mechanism and remain active on both X-chromosomes in females.

**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 organization is *heterochromatin*.

**Folate** A methyl donor obtained primarily from the diet involved in nucleotide synthesis and methylation reactions, including DNA methylation.

**FRAXA** Fragile X mental retardation syndrome involving genetic (CCG repeat expansion) and epigenetic (DNA methylation) changes at the FRM1 gene promoter.

**GABA** γ-Aminobutyric acid, or the chief inhibitory neurotransmitter in the mammalian central nervous system.

**GABAergic inhibitory neurotransmission** Neuronal signaling resulting from the binding of GABA to GABA receptors that decreases the probability of a target cell firing an action potential.

**GAD67** Glutamate decarboxylase (also known as GAD1), an enzyme, which is responsible for catalyzing the production of γ-aminobutyric acid from L-glutamic acid.

**Genome-wide association study (GWAS)** An examination of all or most of the genes in groups of individuals different for some specific trait or disease in order to identify DNA sequence-based factors that contribute to the origin of such phenotypes.

**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).

**Glucocorticoids** Steroid hormones that bind to the glucocorticoid receptor and affect immunological functions, metabolic processes, and responses to stress.

**Glutamatergic excitatory neurotransmission** Neuronal signaling resulting from the binding of glutamate to glutamate receptors that increases the probability of a target cell firing an action potential.

**GR/receptor** The glucocorticoid receptor (GR), encoded by the gene NR3C1, is the receptor that glucocorticoids, such as cortisol, bind to. The GR regulates genes that modulate development, metabolism, immune functions, and responses to stress.

**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.

**Hippocampus** A region of the brain belonging to the limbic system that plays a role in long-term memory and spatial navigation.

**Histone acetylation** Posttranslational 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 methylation** Posttranslational methylation of amino acid residues in histones catalyzed by *histone methyltransferases (HMTs)*. Histone methylation is found at arginine as mono- or dimethylation and lysine as mono-, di-, or trimethylation. Modifications are described depending on the position and type of methylation (mono-, di-, and trimethylation) according to the *Brno nomenclature*. Different types of methylation can be found in either open transcriptionally active or silent (repressive) chromatin (*histone code*). Methylated lysine residues are recognized by proteins containing *chromo domains*.

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

**Histone variants** Variants of canonical histones with distinct amino acid changes accumulating at distinct chromatin regions associated with transcriptional control or silencing.

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

**Hypothalamic–pituitary–adrenal axis (HPA)** A complex interaction between the hypothalamus, pituitary, and adrenal glands that functions to control the stress response and many bodily processes.

**Hypothalamus** A portion of the brain that links the nervous system to the endocrine system via the pituitary gland and controls body temperature, hunger, thirst, sleep, and circadian cycles.

**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 control region (ICR)** Region that shows germ line derived parent-of-origin dependent epigenetic marking which controls the imprinted expression of neighboring imprinted genes.

**Imprinting** See *genomic imprinting*

**In vitro fertilization (IVF)** Fertilization 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.

**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.

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

**Intrauterine environment** The collective conditions affecting a fetus in the uterus.

**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.

**Kinship theory of imprinting** An evolutionary theory, which attempts to explain the origin and evolution of imprinted genes.

**LINE elements** Long interspersed (repetitive) elements dispersed among the human/mammalian genome. LINE elements are usually transcriptional silent and marked by DNA methylation.

**Long-term potentiation (LTP)** A persistent, activity-dependent form of synaptic enhancement of neuron that is a model for certain types of long-term memory.

**Major depression** A mental disorder characterized by low mood accompanied by low self-esteem, suicidal thought, and loss of interest or pleasure in normally enjoyable activities.

**MAPK/ERK signaling** Signaling pathway in the cell, which consists of many proteins, including mitogen-activated protein kinase (MAPK, originally called ERK) that communicates signals from the cell surface to nucleus, affecting the levels and activities of transcription factors and gene expression.

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

**MeCP2** Methyl-CpG-binding protein 2 encodes a protein that is essential for the normal function of nerve cells; mutations in this gene cause Rett syndrome.

**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.

**Monozygotic twins** Twins developed from one zygote that splits and forms two embryos (also known as identical twins).

**MTHFR** Methyl tetrahydrofolate reductase – a key enzyme in the folate (see above)-*S*-adenosylmethionine (SAM, see below) pathway.

**Myelin** An electrically insulating material covering the axons on neurons.

**Neuronal plasticity** The ability of the brain to change as a result of one's experience.

**NMDA/receptor** The *N*-methyl-D-aspartate receptor is an ionotropic glutamate receptor that stimulates intracellular signaling cascades that affect gene transcription, synaptic plasticity, and learning and memory.

**Noncoding RNA (ncRNA)** RNA transcripts that do 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 modeled traits

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

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

**Oligodendrocyte** A type of neuroglia that insulates axons in the CNS.

**Paraventricular nucleus (PVN)** A neuronal nucleus in the hypothalamus containing neurons that are activated by stressful or physiological changes.

**Pituitary** An endocrine gland protruding from the hypothalamus that secretes six hormones involved in homeostasis of an organism.

**PKA** Cyclic adenosine monophosphate (cAMP)-protein kinase A. In LTP PKA is involved in memory consolidation.

**Polyamines** A group of organic compounds that are composed of carbon, nitrogen, and hydrogen, and that have two or more amino groups.

**Polycomb group proteins** Epigenetic regulator proteins forming multiprotein complexes (PRCs = polycomb repressive complexes). Polycomb group proteins possess 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.

**Prader–Willi syndrome (PWS)** A rare pediatric disease caused by chromosomal aberrations or epigenetic misregulation of genes on the paternal chromosome 15.

**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.

**Psychosis** An abnormal condition of the mind, described as involving a loss of contact with reality.

**Reelin** A protein that regulates neuronal migration in the developing brain and also performs various important functions (synaptic plasticity, dendrite development, adult neurogenesis) in the adult brain.

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

**Rubinstein–Taybi syndrome (RTS)** A disorder caused by mutations in the CREBBP gene, characterized by short stature, moderate to severe learning difficulties, distinctive facial features, and broad thumbs and first toes.

***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).

***S*-Adenosylhomocysteine (SAH)** Hydrolyzed 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.

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

**Schizophrenia** A mental disorder characterized by disintegration of thought processes and of emotional responsiveness, involving hallucinations, paranoia, delusions, or disorganized speech and thinking.

**Serotonin** A neurotransmitter produced in the brain that regulates mood, appetite, sleep, and impulse control. It is also known to influence the functioning of the cardiovascular, renal, immune, and gastrointestinal systems.

**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 inhibits 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.

**Skewing of X-chromosome inactivation** Unbalanced inactivation of X-chromosomes in females resulting in different effects of X-linked genetic difference such as recessive mutations.

**snoRNAs** Small nucleolar RNAs involved in processing of small RNAs such as ribosomal RNAs.

**Social environment** Social milieu – the people and institutions – with whom the person interacts.

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

**Spermatogonia** Immature diploid sperm cells which develop into mature spermatozoa (sperm). Major epigenetic changes occur in spermatogonic cells.

**Stem cell** Noncommitted 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.

**Sumoylation** Addition of a Small Ubiquitin-like Modifier or SUMO group to histone residues associated with transcriptional repression.

**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.

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

**Turner syndrome** A disorder affecting women which is caused by a chromosomal abnormality in which all or part of one of the X-chromosome is absent.

**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 noncoding RNA Xist and manifested by various epigenetic modifications including histone methylation, histone deacetylation, and DNA methylation.

**XIC** X-inactivation center. Region at which the XIST-mediated inactivation starts. Allelic changes/differences in the XIC may lead to skewed inactivation.

**XIST** X-inactive specific transcript. The mammalian XIST gene codes for a nonprotein coding RNA that coats the inactive X-chromosome.

**Zebularine** 1-β-D-Ribofuranosyl-2(1*H*)-pyrimidinone, a cytosine analog that can be incorporated in RNA and in DNA, where it has DNA-methylation inhibitor effects.

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