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Sensitive Periods in Epigenetics: bringing us closer to complex behavioral phenotypes

Abstract:

Genetic studies have attempted to elucidate causal mechanisms for the development of complex disease but genome-wide associations have been largely unsuccessful in establishing these links. As an alternative link between genes and disease, recent efforts have focused on mechanisms that alter the function of genes without altering the underlying DNA sequence. Known as epigenetic mechanisms, these include: DNA methylation, chromatin conformational changes through histone modifications, non-coding RNAs, and most recently, 5-hydroxymethylcytosine. Though DNA methylation is involved in normal development, aging and gene regulation, altered methylation patterns have been associated with disease. It is generally believed that early life constitutes a period during which there is increased sensitivity to the regulatory effects of epigenetic mechanisms. The purpose of this review is to outline the contribution of epigenetic mechanisms to genomic function, particularly in the development of complex behavioral phenotypes, focusing on the sensitive periods.

Keywords: Early life adversity, DNA methylation, chromatin remodeling, complex disorders, complex behaviors, therapeutic drugs, suicide.

Introduction:

When the combined efforts of the NIH and Celera completed sequencing of the human genome in 2003, researchers hoped that this code would provide the blueprint for normal development upon which we could decipher the basis of disease. The first large scale genome wide study investigating macular degeneration in 2005 [1], initiated a burst in genome wide association (GWA) studies. However, even with the publication of 1,449 GWA studies thus far [201], and the discovery of numerous genomic variants, relatively simple traits such as height, remain poorly accounted for by genetic variation.

Studies involving twins have been pivotal in the quest to disentangle the genetic contribution of complex traits in classical genetic research. Monozygotic (MZ) twins have identical genetic make-ups, whereas dizygotic (DZ) twins share an average of 50% of their genes. Twin studies of complex behavioral phenotypes, such as schizophrenia and bipolar disorder, have generally found high heritability estimates [2]. However, subsequent mapping and molecular genetic studies have largely failed to identify gene variants accounting for the heritability of these phenotypes [3]. It is possible that this so-called “missing heritability” could be explained by epigenetic mechanisms, which leave DNA sequences unaltered, but may influence a phenotype throughout lifetime.

Epigenetics is the study of factors that can increase or decrease gene expression without directly altering the underlying DNA sequence (for an overview, see Box 1). The functional mechanisms associated with epigenetic regulation are implicated in all levels of physiology from development to chronological aging to disease pathology. These mechanisms include DNA methylation and hydroxymethylation, histone modifications, and small non-coding RNAs. It is generally believed that during certain periods of life the organism may be more susceptible to epigenetic changes. In particular, it is thought that during early life, changes in DNA methylation are more likely to occur.

The purpose of this review is to examine environmental factors associated with epigenetic changes thought to contribute to complex behavioral phenotypes, and discuss whether these occur during sensitive periods.

Sensitive period

The brain responds to life experience by adjusting its neuronal circuits and related functions, including behavior. Collectively, these brain responses to environmental stimuli are referred to as brain plasticity. While the brain always interacts with the environment, brain plasticity is not constant throughout the life cycle. There are periods in life, known as sensitive periods, during which the brain is more sensitive to the effect of experience. There are a number of examples of sensitive periods in the development of diverse brain functions, such as language [4-5], musical skills [6-8] and behavior [9]and there is growing evidence suggesting that epigenetic factors play an critical role regulating molecular mechanisms underlying brain plasticity during these sensitive periods [10-11]. Once defined, epigenetic changes, and in particular, DNA methylation, are thought to be mitotically stable in somatic cells, suggesting that epigenetic patterns are passed down through cell divisions. This stability may explain how events from sensitive periods may influence later-life physiology and disease risk [12].

There are a number of environmental factors which, working alone or in combination, can alter methylation states during sensitive periods, such as embryogenesis and early life. These factors include nutrition, toxins, social environment/maternal care, stochastic events and stress [13]. A classic example is seen in studies looking at the agouti phenotype in mice.

Administering a diet enriched with methyl-donating nutrients to a female mouse during pregnancy, but not prior to pregnancy or after birth, causes her offspring to develop a brown coat, whereas mothers with non-enriched diets, produce offspring with yellow banded coats. Methyl donors are required for both histone and DNA methylation. S-adenosylmethionine (SAM), betaine and

dimethylglycine are examples of methyl donors, defined as substances capable of donating a methyl group (CH_3). A methyl group is transferred from the donor to an acceptor molecule such as the DNA base cytosine or a histone residue (either a lysine or arginine), by the action of a methyltransferase enzyme specific to either the DNA or histone residues. In the case of DNA methylation SAM acts as the donor for DNA methylatransferase enzymes. Nutrients such as folic acid (or Vitamin B_9) and methionine are precursors for SAM, and are thus important in the biochemical process of methylation. In the case of the altered coat colours in mice, the difference is determined by differential methylation patterns at the agouti gene. In the absence of the methyl-donor, the upstream region of the agouti gene in the offspring becomes hypomethylated, resulting in ectopic or artificially induced expression of the agouti gene, and the resulting yellow coat phenotype [14]. Similarly in rats, a protein restricted diet administered to pregnant females resulted in hypomethylation of the hepatic glucocorticoid receptor and subsequent alteration to the metabolic phenotype in the offspring [15]. These are examples of a sensitive methylation period in that, if metabolic disturbances occur during critical time windows of development, the resulting epigenetic alterations can lead to permanent changes in tissue and organ structure or function.

Evidence that early-life may be a sensitive period for epigenetic changes comes from studies investigating maternal care in rodents and non-human primates. These studies have shown that early life environment can influence stress responses in adult rodents [16] and emotional reactivity in adult non-human primates [17]. In 2004, performing a series of experiments on rats, Weaver and colleagues [18] provided the first evidence that the early-life environment, as measured through natural variation in maternal behavior, stably regulates behavioral responses to stress through DNA methylation of the exon 1_7 promoter of the glucocorticoid receptor gene in the hippocampus. This not only confirmed that DNA methylation can be subject to alteration as a result of the social environment, but also supported

92 the existence of a sensitive period for epigenetic regulation of certain biological systems, such as the
93 hypothalamus-pituitary-adrenal (HPA) axis.

94 **Variation in early life-environment: DNA methylation**

95 Following the Weaver et al. (2004) [18] paper, other studies were conducted looking at the
96 effects of early life environment and/or stress on epigenetic regulation of various stress associated
97 genes in addition to the glucocorticoid receptor (GR). These include studies investigating glutamate
98 decarboxylase 1 (GAD1) [19], brain derived neurotrophic factor (BDNF) [20] and arginine vasopressin
99 (AVP) [21], among others. The High licking and grooming maternal care behavior paradigm has also
100 been associated with the development of the GABA system in rat hippocampus through increased
101 expression levels, decreased DNA methylation level and increased histone acetylation levels of glutamic
102 acid decarboxylase (GAD1). [19]. The authors suggest that the increased methylation inhibits the
103 transcription factor NGFI-A from binding to *GAD1*, therefore inhibiting its transcription. Demonstrating
104 that methylation inhibits the binding of a transcription factor adds a level of understanding to the
105 mechanistic role of DNA methylation.

106 To examine the question of early life adversity in an animal model, Roth and colleagues [20]
107 exposed infant rats to stressed caretakers, a paradigm that results in abusive treatment of the infant.
108 The authors observed an increase of DNA methylation of *BDNF* and a decrease in its prefrontal cortical
109 expression. These changes were shown to persist into adulthood. Furthermore, they note alteration in
110 *BDNF* expression and methylation levels in the offspring of the female who experienced early life abuse,
111 suggesting a potential transgeneration effect, as a result of early life stress.

112 Murgatroyd and colleagues [21] found that early life stress resulted in a decrease of methylation
113 in an enhancer region of arginine vasopressin within the paraventricular nucleus (PVN) of the
114 hypothalamus. This decrease of methylation resulted in a persistent upregulation of *Avp* expression in

the PVN, subsequently resulting in a hyperactive hypothalamic-pituitary-adrenal axis. These alterations to stress response sustained their impact for at least 1 year but this altered stress response was reversible using an AVP receptor antagonist. Interestingly, they noted that this region in *Avp* undergoes a decrease in methylation with age in the control mice. However, this age-related hypomethylation is not seen in mice who were subjected to early life stress. This escape of age-shift methylation change, suggests that early life epigenetic alteration may set a new baseline methylation status, which in turn affects the impact of future influences on the epigenome.

Taken together, these findings on early life environment/stress show an important impact on epigenomic properties which are sustained into adulthood. Early in life, neurons are still undergoing changes and reorganization [22] which may provide a window when the epigenome is more susceptible to change. These early life alterations may also influence future epigenomic properties.

Investigating hippocampal tissue and focusing on the promoter of the exon 1_F variant of glucocorticoid receptor gene, an homologous of the rat exon 1_7 variant, McGowan et al. [23] translated the animal findings to humans. Investigating individuals who were severely abused or neglected during childhood and later died by suicide, they found evidence of an association between early-life adversity and glucocorticoid receptor (GR) hypermethylation. In line with the findings in rodents [18] variability in early-life environment in humans showed differences in methylation mapping to equivalent sites of the GR gene. Recently, studies examining methylation levels at the human GR promoter from whole blood found consistent results. One study involving mothers exposed to adversity during pregnancy found increased DNA methylation in the offspring [24]. Another study observed increased GR methylation associated with early-life adversity and linked this to reduced cortisol levels in response to stress testing [25]. Finally in a cohort of subjects with varying levels of childhood maltreatment, Perroud et al[26],

found that levels of promoter methylation of the GR gene varies with the frequency and type of childhood abuse, where severity and frequency were positively correlated with methylation levels.

In a follow-up to the McGowan study, Labonte and colleagues looked at the promoter region of different variants of the glucocorticoid receptor in the hippocampus and the anterior cingulate cortex (ACC) in humans with histories of early life adversity. They showed that total GR expression and three exon 1 variants that are usually highly expressed in the brain (1_B, 1_C and 1_H) were decreased in the hippocampus of abused suicide completers as compared to non-abused suicides and controls. However, in the ACC no differences were found. Analysis of total CpG site-specific DNA methylation levels revealed differences between groups, which were also correlated with respective expression levels. For 1_B and 1_C, an increase in methylation at specific sites was correlated with decreased gene expression, whereas for 1_H, a decrease in methylation at a specific site was correlated with a decrease in gene expression [27]. This study concluded that methylation levels at these non-coding promoter regions also influence the expression of GR in the hippocampus, but not in the ACC.

Another study by Labonte et al., investigated whole-genome promoter methylation patterns associated with early-life adversity [28]. They used a technique known as methylated-DNA immunoprecipitation (MeDIP), where an antibody against methylated cytosine captures methylated regions of the genome coupled with an array containing ~400, 000 probes covering the all predicted human promoter regions. The study shows a clear difference between the methylation patterns in abused suicide completers versus non-suicide controls in the hippocampus, notably showing an overrepresentation of hypermethylated promoters in abused suicide completers. Furthermore, at the genome level, promoter region methylation inversely correlated with gene expression level. Follow up work, identified among the differentially methylated genes, an overrepresentation of genes involved in

neuroplasticity and found that these changes were primarily taken place, among the genes investigated, in hippocampal neurons rather non-hippocampal cells.

Dynamic DNA methylation: Demethylation

In light of this research discussed above, scientists began questioning whether or not epigenetic reprogramming occurs only during sensitive periods such as early-life or whether organisms remain susceptible to these environmental factors for their entire lives. Post-mitotic cells like neurons are thought to maintain very stable covalent modifications, like the methyl-group added to a cytosine, indefinitely as the DNA from these cells is not generally recycled or replicated [29] (with the exception of neurons from more plastic brain regions). However, in animals, it is known that behavioral phenotypes associated with negative early-life environments may change following positive environmental exposure. For instance, environmental enrichment may reverse behavioral phenotypes associated with low licking and grooming in rats [30]. While it is not clear if the effects of environmental enrichment are mediated through reversal of epigenetic changes previously acquired or through other changes, certain evidence suggests that DNA methylation may be more dynamic than previously thought. Early evidence that DNA methylation status could be altered later in life can from a study examining patients with kidney failure [31]. As kidney failure is accompanied by hyperhomocysteinaemia, which results in an increase in serum homocyteine levels, a powerful inhibitor of SAM mediated methyltransferases, the authors were interested in the impact of hyperhomocysteinaemia on DNA methylation levels. They found an overall decrease in methylation which was associated with alter gene expression. Interestingly, the common treatment of folate administration reduced the level of serum homocysteine and corrected DNA hypomethylation. These results suggest that DNA methylation has a dynamic capacity beyond an early life sensitive period. In the same vien, Weaver et al found that methionine (a methyl-donor) treatment alters methylation at the GR promoter, resulting in a significant hypermethylation in that

region relative to the vehicle control [32]. This alteration in the methylation pattern translated into an altered stress response in animals that already had established stress responses. Recently, a number of studies investigating pharmacological agents such as antidepressants [33-34] or neuronal activity itself [35] have reported that methylation patterns are dynamically regulated, suggesting that DNA methylation acquired as a result of environmental factors may potentially be reversible.

The idea that DNA methylation is dynamic in a post-mitotic neuron, transient not only on a scale of years but possibly minutes [35], raises the question of what molecular mechanisms may facilitate such processes. Over the last 30 years, we have learned a great deal about how methyl groups are added to cytosines (see [36] for a review on DNA methyltransferases). However, the process of DNA demethylation in mammals remains unclear. Though active demethylation is known to occur in certain contexts such as in embryogenesis before imprinting [37], it remains contentious whether active demethylation exists in post-mitotic neurons or if it is a passive process resulting from the dysfunction of DNMT1. DNMT1 is the enzyme which normally maintains the methylation patterns by targeting hemimethylated sites and transferring a methyl group to produce a fully methylated site [38]. As an active process, demethylation might require either a direct enzymatic cleavage or it could be the result of a substitution/DNA repair mechanism. On the other hand, in the related field of histone research, demethylation is well understood. In 2005, Metzger et al described a family of histone demethylases called lysine specific demethylase (LSD1), which are capable of removing the methyl group from mono- and di- methylated lysines, specifically histone 3, lysines 4 and 9 (H3K4, H3K9) [39]. Subsequently, Tsukada et al discovered that a specific JumonjiC (jmjC) domain-containing protein, JHDM1 (JmjC domain-containing histone demethylase 1), is capable of mediating lysine demethylation from all three methylation states [40]. It is thus tempting to speculate that similar mechanisms should exist for DNA. While the lysine methylation of histone tails occurs on a nitrogen atom, the cytosine methylation of DNA occurs on a carbon atom, and the carbon-carbon (c-c) bond is thermodynamically very stable. The

recent discovery of 5’-hydroxymethylcytosine (5'-hmC) [41] may provide an answer to this question. The oxygen atom in 5'-hmC is electronegative enough to in theory, destabilize the c-c bond, making it an attractive candidate as an intermediary in the demethylation process [42-44].

Epigenetics and Behavior

The dynamics of epigenetic alterations have provided us with insight into the development of a number of behaviors and mental disorders, such as learning and memory, anxiety, stress responses, depression and other psychiatric disorders. These phenotypes have been extensively examined in both animal models and humans.

Animal models of learning and memory have revealed the importance of histone acetylation for the initiation of new gene expression which is required for the stabilization of learned information into long term memory [45-47]. For example, using a transgenic mouse model to knock down CREB binding protein (CBP) associated histone acetyltransferase activity, it was demonstrated that long term memory can be recovered by administering a drug that stops the activity of histone deactylases [47]. The authors suggest that, independent of the CBP activity, acetylation at specific gene targets could act to prolong the window of transcription by covalently opening the chromatin structure. Likewise, environmental enrichment has been shown to recover chemically induced memory impairments in mice through a general increase in histone acetylation and lysine 4 methylation [48], which are both epigenetic marks associated with active transcription.

Genes such as *Bdnf* [49-50], *Reln*, *Ppp1ca* [51] and *Hes5* [52] are shown to be epigenetically regulated in the consolidation of fear memory. Fear conditioning in mice demonstrates that dynamic DNA methylation regulates the expression of the synaptic plasticity gene *Reln*, and memory suppressor

gene *Ppp1ca* [51]. The environmental context of fear learning, increases the levels of DNMT1 in the hippocampus, which associates with targeted increases of methylation of *Ppp1ca*, decreasing its expression. This is associated with decreased methylation in the promoter of *Reln*, which is concordant with its increased gene expression in the context of fear learning. Interestingly these methylation changes were shown to return to baseline 24hr after fear conditioning, suggesting highly dynamic DNA methylation changes in adult mice.

Animal models commonly use stress response as a corresponding phenotype for depression and anxiety. Not surprisingly, the epigenetic research in stress response has focused on genes and proteins associated with the hypothamlamic-pituitary-adrenal axis such as the *Gr* [32, 53], corticotrophin-releasing factor (*Crf*) [33], *Bdnf* [20], estrogen receptor-alpha1b (*Erα1b)* [54] and nerve growth factor-inducible protein a (NGFI-A) [55]. These studies show that using various stress paradigms including maternal care and intruder avoidance in animal models, induces epigenetic changes, that results in altered gene expression. In the case of *Crf*, the authors showed that stress in adulthood is associated with a decreasing in methylation in the cAMP-responsive element (CRE) binding site. The absence of methylation enabled CRE to bind, inhibiting transcription of C*rf*. This demethylation was associated with social avoidance behavior but both the methylation and behaviour where recovered with the administration of an antidepressant [33]. This study presents a link between the environment leading to a molecular alteration which associates with a behavioral change, and importantly, the potential reversal of these molecular and behavioral phenotypes with chemical therapeutic intervention.

Animal models provide us with tremendous insight into complex phenotypes such as memory, stress response and depression, but ultimately, it is important to confirm these mechanisms in humans. In order to assess epigenentic alterations in humans, researchers have looked to proxy tissue such as leucocytes from whole blood, to assess alterations to chromatin confirmation and DNA methylation

252 patterns. Some of these studies have been describes in an earlier section and others examples
253 include,,human umbilical cord blood used to show that maternal depression in the third trimester was
254 associated with increased methylation levels in the analogous gene region of neonatal babies [56].
255 These results were associated with increased cortisol stress response at 3 months. The authors conclude
256 that increased infant stress responsivity is associated with 3 trimester maternal depression, which is
257 likely mediated by the increase *GR* promoter methylation. Another study further demonstrated that
258 hypermethylation in *GR* from stress sustained during pregnancy is maintained in the offspring beyond
259 infancy to adolescence [24]. Recently, it was shown that antidepressant use in patients with major
260 depressive disorder increased levels of *BDNF* in the periphery [57]. Specifically, an association was
261 shown between the level of H3K27me3 levels and patients who responded to drug treatment. The
262 decrease of this transcriptionally repressive mark was associated to an increase *BDNF* in responders
263 only. Moreover, there was a significant inverse correlation between depression scores and *BDNF* levels.
264 This suggests behavior can be mediated epigenetically by external factors however some predisposing
265 factor is required to engage the epigenetic machinery required for change. Human blood has also been
266 used to assess DNA methylation changes in other mental disorders such as posttraumatic stress disorder
267 [58], bipolar disorder [59-60] and autism [61].

268 The use of blood to detect DNA methylation or chromatin conformational changes is debatable
269 in that it remains unclear to what extent developmental epigenetic changes acquired postnatally are
270 cells specific or generalized in the organism, though some studies have tried to demonstrate similarities
271 between cells derived from peripheral tissue with those found centrally (see box 2).

272 Studies using post-mortem brain tissue have been used to implicate DNA methylation alteration
273 in *RELN* [62-63], *SOX10* [64], *GAD1* [65] in schizophrenia. Similarly, post-mortem brain tissue from the
274 prefrontal cortex (PFC) have linked epigenetic mechanisms to suicide and depression through the

investigation of *SSAT* [66], *GABA$_A$R* [67],,*BDNF* and its high affinity receptor tyrosine kinase type B (*TRKB*) [68].

In 2003, Dwivedi et al showed that both *BDNF* and *TRKB* mRNA levels were reduced in the prefrontal cortex and hippocampus of suicide completers [69]. Keller and colleagues later demonstrated that DNA methylation at the *BDNF* promoter (specifically at exon IV) is increased in the Wernicke's area in suicide completers, and this high level of methylation was associated with a decrease in BDNF expression. This was also shown to be site-specific to the *BDNF* promoter as genome-wide methylation levels showed no between-group differences [70]. A study focusing on the truncated form of the TRKB, which is the BDNF receptor, indicated increased methylation at two sites in the frontal cortex of suicide completers as compared to controls [68]. The increase in DNA methylation associated with decreased expression in the frontal cortex, but not the cerebellum [68], suggesting at least some level of regional specificity.

TRKB expression in suicide was also shown to be regulated by histone modifications. Specifically enrichment of H3K27 methylation was found at the promoter regions of the truncated form of the *TRKB* receptor (*TRKB.1*) [68]. This increased histone methylation was associated with decreased expression; however differences in DNA methylation were only seen in the frontal cortex (BA10) and not the cerebellum. Additionally, in line with the study on peripheral *BDNF* levels in antidepressant drug responders [57] previously described, a study using suicide brains found that antidepressants use had the same association of decrease in methylation at H3K27 and an increase in *BDNF* expression [71],again suggesting an influence of therapeutic drugs on histone modifications in humans similar to that seen in rodents [72].

MicroRNAs have also been described in the context of suicidal behavior and other affective disorders. Smalheiser and colleagues [73], found gobally down-regulated miRNA expression levels in the

298 prefrontal cortex of depressed suicide subjects not using antidepressants. The authors conclude that a
299 network of down-regulated miRNAs can affect various genes which have been previously associated
300 with depression and suicide. The same group also recently published a review examining the role
301 miRNAs in neurogenesis, synaptic plasticity, pathological stress changes, major affective disorders and
302 suicidal behavior, see ref[74].

303 An overview of papers published in behavioral epigenetics can be found in the table below:

304

Table 1: Behavioral Epigenetic Studies

Behaviour	Epigenetic Mechanism	Studied in	Brain region	Gene	Period	Reference
Learning and Memory	Ch- R	Mouse	HPC, Cortex		EL	[48],[47]
	His-Ac	Mouse	HPC			[45-46]
	DNA meth	Mouse	HPC	*Ppp1CA*, *Reln*	Adult	[51]
Fear/Aniexty	DNA meth/ Ch-R	Rat	HPC	*Bdnf, Hes5*		[50, 52]
	Ch-R	Mouse	PFC	*Bdnf*		[49]
	His-Ac	Rat	HPC	Transcriptome	EL/ MC	[75]
	DNA meth	Human	Blood	GR		Redkte 2011 [25]
Depression	DNA meth	Human	Blood	GR	Prenatal/dev	[56]
			Cortex	QKI		[76]
	His-Meth		Blood	*BDNF*	Adult	[57]
	Ch-R, His-Meth	Mouse	HPC	*Bdnf*	Perinatal	[77]
	His-Meth				Adult	[72]
Stress response	DNA meth	Mouse	HYP, AMY, STR,PVN	*Gr Gdnf,Crf*	Perinatal/ EL	[33-34, 53]
			Forbrain	KAPI	Adult	[78]
		Rat	HPC, MPO, PVN	*Gr, ERα1b,,Bdnf, Avp*	EL/ MC	[21, 32, 54, 75, 79-82]
Suicide	DNA meth	Human	HPC	GR, rRNA	EL	[23, 27-28, 83]
			PFC	*BDNF, TRK.B, $GABA_AR$, SSAT*		[66-67, 84-85]
	His-meth		PFC	Polyamines		[86]
	miRNA		Global	See refs		[74][73]
Post Traumatic Stress	DNA meth	Rat	HPC	*Dlgap2,Bdnf*		[87-88]
		Human	Blood	*TRLs, ILs*		[58]
Psychotic disorders	DNA meth	Human	PFC, blood	*RELN, MEK1, BDNF, SOX10, GAD1*	Adult	[59, 62-65, 89]
	His-meth		PFC	*GAD1,2,NPY, STT*	Adult	[90]
	miRNA		Blood	miR-130b		[91]
Autism	DNA meth	Human	Brain, Blood	X chromosome	Adult	[61]

Abbreviation: DNA-meth, DNA methylation; Ch-R, Chromatin Remodeling; His-meth, Histone methylation; His-Ac, Histone Acetylation; AMY, Amgydala; HPC, Hippocampus; HYP, Hypothalamus; PFC, Prefrontal Cortex; PVN, paraventricular nucleus; STR, Striatum; DAT, Dopamine transporter; EL, Early Life; MC, Maternal Care

307

Conclusions

Over the last decade, we have begun to learn how the social environment has affected our genome through epigenetic modifications, eventually leading to behavioral regulation. Gradually, we are building an intricate molecular picture of epigenetic changes associated with complex phenotypes, yet many questions still remain, including, among many others, to what extent, and which, DNA methylation changes are stable or dynamic, whether environmentally induced DNA methylation is inheritable, and if so, how does it affect future responses to environmental factors.

As we move forward understanding how epigenetics modifies behavior and mental disorders, it will be important to control for the influence of age (box 2), environment, genetic sequence (box 2), and to gain insight into how epigenetic effects impact different CNS cells types, as well as how these correlate with peripheral tissues. A number of efforts are underway to develop epigenomic reference maps in both healthy and disease populations, which will undoubtedly provide important resources to better understand how these factors interact.

Epigenomics is a young research filed and there is a significant need to increase our mechanistic understanding of epigenomic processes. As we continue to develop tools and resources, we will come closer to potential therapeutic interventions. Similarly, better understanding how the environment affects us, particularly in early life, may promote more appropriate preventive measures, potentially altering our approach to managing complex diseases and behaviors.

Future Perspectives

As we continue to develop tools and resources in the field, we come closer to therapeutic applications on a molecular level. The impact of environmental interactions such as maternal care, on the development of DNA methylation patterns suggests the possibility of non-chemical interventions. However, just as genes rarely have binary influence on a trait, neither do epigenetic modifications. Elucidating the interactions of epigenetic modifications with other epigenetic modifications as well as with the recruitment of numerous additional proteins, that ultimately constitute the activation or repression complexes in transcription, is an important future goal within the field. The continued development of reference databases which model healthy epigenomic states will help us piece together these elaborate interactions bringing us closer to molecular definitions of complex disease phenotypes.

Up to now, we have considered the environment's impact on the epigenome and the downstream consequences of alterations to DNA methylation patterns, chromatin conformational changes and miRNA expression. We have started to piece together how these various epigenetic mechanisms alter gene expression but very little of our focus has been directed toward the presumably hormonal changes are capable of initiating methyltranserase proteins and how protein complexes such as the polycomb-group proteins might direct this methylation pathway to a specific gene and region. Within the next few years it will be of great importance to fill in this missing link in the epigenetic pathway.

Executive Summary

Sensitive Period

- **A period of undefined duration often associated with development and early life, during which the organism is more susceptible to epigenetic changes through environmental influences.**

Early life adversity

- **Refers to negative early life events such as physical or sexual abuse, as well as parental neglect.**

Variation in early life-environment: DNA methylation

- **Early life adversity has been associated with dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis.**
- **Animal and human studies suggest that the early environment may regulate HPA axis function by promoting DNA methylation changes. It is believed that these epigenetic changes are responsible for altered stress reactively and could underlie psychopathologies.**

Dynamic DNA methylation: Demethylation

- **Early life behavioral phenotypes associated with early-life environment may change following positive environmental exposure.**
- **A number of recent papers have shown that DNA methylation can be dynamic and is not necessarily confined to a sensitive period.**
- **The dynamic nature of DNA methylation suggests that DNA demethylation must be an active process.**
- **As no function has been associated with the recently discovered 5'-hydroxymethylcytosine, some researchers believe it may be the intermediary between a methylated and non-methylated cytosine.**

Epigenetics and behavior

- **Histone acetylation is important for new gene transcription in the formation of long-term memories.**
- ***Crf* was shown to be epigenetically modified by stress in adult mice resulting in altered stress response and both the molecular mechanism and behavior were recovered using an antidepressant.**
- **In humans. blood and post-mortem brain tissue have been used to suggest the involvement of epigenetic regulation of a number of genes including: *BDNF, GR, RELN, MEK1, SOX10, GAD1 GAD2, NPY, STT, SSAT, TRKB.***

346

Box 1

Development of Epigenetics

The term epigenesis was first coined by Conrad Waddington in 1941 and used to describe epigenetics as a sort of place where the environment and the genome intersected to produce phenotypes [92]. Waddington [92] likened epigenetics to “canals” in a “phenotypic landscape” which could direct phenotype based on interactions with the environment early in the life of an organism. Canalization allowed for how genetically different starting points could be “funneled” to a common endpoint or phenotype. Alternatively, “decanalization” described directing a common genetic start point to different endpoints resulting in phenotypic variation. Experiments in development, both in plants [93] and animals [94] illustrate Waddinton’s theory, demonstrating for example that a protein can act as a canalizing agent by directing underlying genetic variability to a common phenotype (canalization) but in its absence or dysfunction, phenotypic variation occurs (decanalization).

Expanding Waddington’s seminal position in the field, Holliday and Pugh, shifted epigenetics from a metaphysical concept to a tangible molecular mechanism, specifically by describing DNA methlyation in cancer in 1975 [95]. Though the DNA methyl mark was originally discovered in 1925 [96] and subsequently characterized in the 50s [97], its functional role in gene expression was only discovered with work on X-chromosome inactivation [98] and in cancer [99].

DNA methylation is the covalent bond of a methyl group (-CH3) to the 5' carbon of a cytosine residue. In mammals, this is almost exclusively found in the context of a cytosine followed by a guanine (CpG). Moreover, CpG sequences are not evenly dispersed throughout the genome. Conversely, they are known to concentrate in regions referred to as CpG islands. CpG islands are variable in length (~200bp), rich in CG content, tend to be methylated less frequently than CpG dinucleotides found outside of island, and correspond to promoter regions for 50–60% of human genes [100]. Generally, DNA methylation represses gene transcription in two opposing manners; either by attracting repressing proteins [101] or by blocking transcription factors from binding to the promoter sequences [102]. CpG methylation is carried out by a family of enzymes known as DNA methyltransferases (DNMTs). Certain classes are responsible for the maintenance of DNA methyl states (DNMT1 and DMNT2), whereas others are responsible for de novo CpG methylation (DNMT3A and B) [103-104].

Regions with low levels of methylation are associated with the transcriptionally permissive open chromatin (heterochromatin) conformation [105]. Chromatin is the DNA-protein complex found within the nucleus of a cell, and responsible for the organization of DNA. It is made of a core unit called a nucleosome composed of ~143 base pairs of DNA wrapped around a histone octamer. Histones are basic proteins with an N-terminal “tail” which protrudes from the nucleosome and can be subject to a number of covalent modifications. These modifications include: acteylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination and sumoylation. Chromatin modifications were initially investigated in the context of posttranslational protein modifications independent of epigenetic mechanisms but the close interaction with DNA methylation suggested an epigenetic function [106] and their role is being implicated in many behavioral phenotypes including mood disorders [107]. For a complete overview of these modifications [see 108].

The idea that there are many ways to modify histones and many possible interactions between them became the precursor for what has now been termed the histone code. This concept first coined in 2000 [109] describes reversible covalent modifications to histones which can act in particular combinations or alone to influence transcriptional activation or repression. Certain modifications are strongly associated with transcriptional activation and repression, such as Histone-3 lysine- 4 tri-methylation (denoted H3K4me3) and histone- 3 lysine- 27 tri-methylation (H3K27me3), respectively. On those occasions where these marks do not result in the expected transcriptional activity, the histone code offers a potential explanation.

348 **Box 2:**

Cell Specificity

Whether epigenetic modifications occur in a cell specific manner is an important question which researchers have been trying to address for several years. Different cell types are believed to have different methylation pattern, this is because cell fate is directed by DNA methylation patterns [110-112]. This implies that on a cell-to-cell basis, the patterns are unique There have been a number of studies which have examined DNA methylation patterns in proxy tissues such as leukocytes from pancreatic cancer patients [113], saliva from diabetic patients [114], whole blood DNA from patients with bipolar disorder or schizophrenia and their discordant MZ twins[60] and mononuclear cells in healthy subjects as a measure of prefrontal cognition and activity [115]. Though there is plenty of evidence for cell specificity in epigenetic patterns, disease-associated epimutations have been identified across tissues. For example, the same genomic region was found to be hypomethylated in post-mortem brain tissue from people with psychosis as compared with leukocyte DNA taken from their MZ twins with psychosis[60]. Other studies have shown similar results, such as a consistent difference in DNA methylation of the HLA complex group 9 genes across multiple tissues in bipolar disorder [116]. On the other hand, there are many studies that have shown that there are important differences of DNA methylation across cell types. For example, one study showed differences in the methylation patterns of neuron-specific genes in neuronal cells compared with glial cells [117]. Because epigienetic status influences gene expression, it is logical that certain genes could have a specific DNA methylation pattern depending on cell type. In cell differentiation, certain genes such as Nanog and Oct4 are critical in differentiation from a pluripotent state to a defined state. In pluripotent cells, their promoters are unmethylated however when the cell is differentiated and these genes are no longer required, their promoters become methylated [118]. These data show that epigenetic states depend on the cell type and the function of the gene within that cell. That said, the dysregulation of a gene in a disease state may have an impact across several tissues and cell types which may be independent of some of the cell-specific methylation or epigenetic patterns. With the advancement in technology and the increase in motivation to sequence methylation patterns at a genome-wide cell specific level [119-120], there may soon be an answer to this question.

DNA sequence

Very recently, it was shown that the underlying DNA sequence of a gene has an important role in determining the methylation pattern within a cell. Using embryonic stems cells and derived neuronal progenitors cells from mice, Lienert and colleagues [121] showed that endogenous DNA methylation patterns are attributable to methylation-determining regions of DNA sequence, which direct DNA methylation to specific sites within the gene. Particularly, they showed that a proximal promoter is able to guide its methylation state in a pluripotent cell and undergo the expected reprogramming in a differentiated cell. This finding demonstrates an interplay between the underlying genome and DNA methylation patterns, which may help to elucidate the mechanisms involved in erroneous patterns in disease states. This is not the first scenario which describes an interplay between sequence and methylation. It is known that methylation plays an important role in silencing repeat sequences and in dosage compensation, even in a non-X inactivation manner for example, in mediating allelic imbalance [122].

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Box 2 Con't

Chronological Age

Research on the process of aging, shows that the methylation pattern of certain genes, in healthy individuals, change as an individual ages [123-124]. There are a number of longitudinal studies attempting to discern the intra-individual effects of age on methylation and how these differences may be physiologically important [125-126]. Aging is associated with stochastic dysregulation of gene expression, resulting in divergent transcriptomes [127]. This suggests that as we age, we are already subjected to random mutations which influence gene expression. In general, aging is associated with global hypomethylation and regional hypermethylation [128].Though this sounds contradictory, it simply describes that overall, there is less methylation in gene body or repeat regions; areas that are normally expected to be methylated. However, certain specific genes such as tumor suppressor genes are found to have higher levels of methylation [129]. Furthermore, hypomethylation is also associated with age related diseases such as, Alzheimer's, cancer, atherosclerosis and other neurodegenerative diseases [130-131]. This suggests that hypomethylation may be a normal consequence of natural aging, and the dysregulation of this process may be related to age-associated pathologies. Though many mechanisms have been proposed for this decrease in methylation, such as deficient DNMT1 function, the causes remain unknown [128]. Nonetheless, it is important to take into account that alterations in DNA methylation patterns and chromatin conformation via HDAC/HAT mechanisms [132] occur with age and may have impact on age related pathologies, and influence the interpretation of experimental results.

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