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Chapter *in* Advances in Experimental Medicine and Biology · May 2017

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Anxiety and Epigenetics

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Andrew A. Bartlett, Rumani Singh, and Richard G. Hunter

Abstract

Anxiety disorders are highly prevalent psychiatric disorders often comorbid with depression and substance abuse. Twin studies have shown that anxiety disorders are moderately heritable. Yet, genome-wide association studies (GWASs) have failed to identify gene(s) significantly associated with diagnosis suggesting a strong role for environmental factors and the epigenome. A number of anxiety disorder subtypes are considered "stress related." A large focus of research has been on the epigenetic and anxiety-like behavioral consequences of stress. Animal models of anxiety-related disorders have provided strong evidence for the role of stress on the epigenetic control of the hypothalamic-pituitary-adrenal (HPA) axis and of stress-responsive brain regions. Neuroepigenetics may continue to explain individual variation in susceptibility to environmental perturbations and consequently anxious behavior. Behavioral and pharmacological interventions aimed at targeting epigenetic marks associated with anxiety may prove fruitful in developing treatments.

Keywords

Anxiety • Epigenetic • Stress • Glucocorticoid • Plasticity • Hippocampus • Amygdala • Prefrontal cortex • Histone • DNA methylation • Noncoding RNA

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R. Delgado-Morales (ed.), *Neuroepigenomics in Aging and Disease*, Advances in Experimental Medicine and Biology, DOI 10.1007/978-3-319-53889-1_8

8.1 Introduction

Anxiety disorders (ADs) are among the most common psychiatric disorders, occurring in roughly a third of the US population. They are also highly comorbid with depression and substance abuse disorders, and the pathogenesis of AD is likely highly interrelated [1]. While anxiety disorders are heritable and genetic factors play a role in anxiety disorders, most of the risk of these disorders is environmental in nature [2]. Stress, particularly in early life, substance abuse, circadian, and microbiota have all been shown to have an influence on risk of anxiety disorders [3–6]. Further, it is likely that anxious phenotypes are influenced by more subtle factors such as the interplay between an anxious parent and a child whose early life is defined in part by adapting to that parent's behavior [7, 8]. Indeed the latter case is emblematic of one of the important distinctions between heritability, which can include epigenetic mechanisms, both behavioral and molecular, and the strictly genetic inheritance with which heritability, in general, is often conflated. ADs are moderately heritable with most of the disorders in the classification showing heritability in the range of 30% [9]. GWASs have generally not met the criterion of genome-wide significance, and candidate gene approaches have also been relatively unsuccessful [10]. However some genetic polymorphisms do show replicable associations with AD, for example, the glucocorticoid receptor chaperone FKBP5 has been associated with risk of post-traumatic stress disorder (PTSD) in individuals with a history of child abuse in an African–American sample, and the same sample also demonstrated a female-specific association with PTSD and the ADCYPAP1R1 receptor for the neuropeptide PACAP [11, 12]. The catechol-O-methyltransferase (COMT) valine158methionine polymorphism has been repeatedly implicated in risk of panic disorder, though with different alleles imparting risk in European versus Asian populations [13]. Even these findings point to the role of other contextual factors like ancestry and sex as influences on the underlying genetics, developmental context also appears to influence the expression of genetic risk, as a study in a Swedish cohort has shown that different risk factors act at different times across adolescence and early adulthood [14].

While environmental factors like stress are clearly significant in many anxiety disorders, their effects can vary wildly across individuals. The role of the environment is most clear with PTSD, where most individuals are resilient and only a fraction go on to develop the disorder after a trauma exposure [15, 16]. The question of differential susceptibility in AD is another to which genetic explanations thus far fall short.

8.2 The Neuroanatomy of Anxiety Disorders

Human anxiety is defined by emotional symptoms as well as behavioral and physiological phenotypes. Much of the work in understanding the underlying neuroanatomy involved in anxiety-related pathology has been done using animal models. Specifically, the focus has been on conserved endocrine systems and brain regions

that identify or respond to environmental threats. For example, noxious stimuli may result in freezing behavior, sympathetic nervous system activation, and subsequent endocrine response in both the rat and the human. Across species, the limbic system and the prefrontal cortex appear to be crucial for regulating threat recognition and response. The hormonal response to threat appears, likewise, remarkably similar and feature highly conserved signaling pathways.

Within the limbic system are a number of structures necessary for threat response and assessment. The amygdala, for instance, appears to be necessary for fear responses. Patients with Urbach-Wiethe disease have compromised amygdala function and report loss of feelings of fear [17]. In rodents, fear conditioning models pair a benign stimulus, the conditioned stimulus (CS), with a noxious stimulus, the unconditioned stimulus (US). A frequently used example of a US is a foot shock which elicits freezing behavior, an unconditioned response (UR). After pairing of the CS with the US, the CS alone can elicit this freezing behavior. This freezing is referred to as the conditioned response (CR). Lesioning the amygdala has been shown to obliterate freezing behavior, the CR, in conditioned rats [18, 19]. Stimulation of the amygdala during CS presentation produces subsequent freezing behavior to the CS without US pairing [20]. During encoding of fear memories, hippocampal inputs to the amygdala appear to be necessary for CS-US pairing for contextual clues [21]. Within the amygdala, various subnuclei have been shown to regulate different processes. The central amygdala (CeA) appears to regulate CR expression through projections to the periaqueductal gray (PAG) [22]. The lateral amygdala (LA) appears to receive CS and US inputs through cortical and thalamic innervations [23]. The stimulation of a subpopulation of neurons in the LA, when paired with presentation of the CS, appears to be sufficient to generate a CR. The basal amygdala (BA) appears to have a dual role in both CR expression and suppression [24]. Two distinct populations of neurons were identified in the BA, one innervated by the hippocampus and the other innervated by the PFC [24, 25]. During extinction of the CS-US pairing, the PFC appears to inhibit the BA and attenuate freezing CR [26–28]. The pairing of the CS-US improves prediction of the US allowing for rapid behavioral response. However, when the CS fails to correctly predict the US, the association must not continue to persist else anxiety or avoidance for the US has now generalized to benign stimuli. These regions are critical to avoidant and anxiety-like behavior. The dysregulation of these circuits may lead to recurrent avoidance or anxious behavior to inappropriate stimuli similar to the definition of human anxiety.

8.3 The Neuroendocrine Axis in Anxiety Disorders

The HPA axis is a critical component of the acute stress response. In response to a stressor, the body must divert resources appropriately in order to efficiently address the challenge at hand. In part, this tentative balance is achieved through the activation of the HPA axis. The HPA axis is a negative feedback loop that begins with the release of arginine-vasopressin (AVP) and corticotrophin-releasing factor (CRF)

into the pituitary portal from the paraventricular nucleus (PVN) in the hypothalamus. This release promotes the production of proopiomelanocortin (POMC) in the pituitary. POMC is subsequently converted to adrenocorticotropic hormone (ACTH) and released into the bloodstream. The adrenal gland produces corticosteroids in the adrenal cortex as a consequence of ACTH. Corticosteroids are released into the blood and bind to the mineralocorticoid (MR) and glucocorticoid receptor (GR). In the PVN, pituitary and hippocampus GRs inhibit the production of CRF resulting in negative feedback loop.

The adrenal gland also produces two other hormones, epinephrine and norepinephrine, from the adrenal medulla in response to ACTH. These hormones do not engage in a self-regulating negative feedback loop but are indirectly regulated through the actions of GRs. The role of these hormones is to control the response of the body and the peripheral nervous system, for instance, reducing digestion and immune function while increasing heart rate and blood pressure acutely. Interestingly, pharmacological interventions targeting norepinephrine receptors have proved effective reducing phobias during fear memory reconsolidation [29]. These findings suggest that the autonomic nervous system may remain a potential area for research and intervention in stress-related anxiety disorders such as phobias and PTSD.

8.4 Epigenetic Factors

The relative prominence of the environment and the moderate contribution of genetic factors to the pathogenesis of anxiety disorders have made the study of these disorders through the lens of epigenetics a fruitful avenue of research in recent years. Many molecular epigenetic mechanisms have now been implicated in AD, including DNA and histone modification as well as noncoding RNA (ncRNA). Epigenetics, in the strict molecular sense, refers to regulation of DNA sequences that does not involve alteration of actual base composition. Transcription and other genomic functions are regulated directly through epigenetic modifications that typically annotate DNA and its associated histones via acetylation, methylation, and phosphorylation. These epigenetic marks are tightly linked to chromatin state as complex of DNA, RNA, and protein. Open chromatin is associated with active transcription, whereas closed chromatin is associated with transcriptional silencing. Epigenetic marks that define the epigenotype include DNA methylation and various modifications (e.g., methylation, acetylation) of histone proteins that are complexed with DNA. DNA methylation occurs at cytosines of CpG dinucleotides and is catalyzed by enzymes of the DNA methyltransferase family. DNA methylation may inhibit gene expression by direct interaction with factors that repress transcription or, indirectly, through recruitment of methyl-CpG binding proteins (MeCP2 and MBDs) complexed with enzymes that modify histone proteins. These modifications can transform chromatin from an active to a repressed state, or vice versa.

The role of the epigenome in etiology of anxiety disorders and variations in behavior and neurological status can now be investigated. Of particular importance in epigenetics research is the fact that epigenetic marks are modifiable both in the

germ line and in somatic tissues by genetic, environmental, and stochastic factors. Each cell in the human body possesses not only a genotype, identical in all somatic cells of an organism, but also an epigenotype that is highly variable among the different tissues of an individual. Errors or alterations in epigenotype can occur as primary stochastic events or secondarily in response to either genetic mutations (e.g. transposition events) or environmental exposures. Therefore a discussion of potential epigenetic etiologies of anxiety disorders necessarily involves both genetic and environmental factors. Dysregulation of genes that control epigenetic mechanisms leads to a number of "epigenetic syndromes" falling into two groups. Those with changes in genes regulating epigenetic marks include enzymes such as DNA methyltransferases, methyl-binding proteins, and enzymes that affect histone modification. The second category involves genes that are regulated by epigenetic marks, for example, imprinted genes.

8.5 Epigenetics in Animal Models of Anxiety

Twin studies of generalized anxiety disorder have failed to identify either a genetic basis for or strongly heritable component of the disorder [30]. This class of mental health disorders is often comorbid with addiction [31]. Both involve pathological behaviors that have a neurobiological basis. Over the last decade, increasing focus has been placed on how gene-environment interactions mediated by epigenetic molecular mechanisms might improve our understanding of the disease. Though environmental influences including trauma and substance abuse are known contributors to anxiety, it is difficult or impossible at present to examine molecular epigenetic changes in the central nervous system of clinical populations, and given the tissue-specific nature of epigenetic mechanisms, accessible peripheral tissues such as blood or epithelial cells may not reflect the changes present in the brain. For these reasons, animal models have been employed to mimic the signs of anxiety. While symptoms, such as intrusive thoughts, are impossible to model in rodents or nonhuman primates, sophisticated paradigms have been used to model aspects of social anxiety, general anxiety, and more broadly anxious temperament. In rodent models, common behavioral paradigms to assess anxiety-like behaviors include the elevated plus maze (EPM), light/dark box (LD), open field test (OFT), social defeat (SD), and the social interaction test (SIT). The EPM consists of two arms of open platforms and two arms of closed platforms featuring three walls. The EPM is based on an innate fear of heights and open spaces such that rodents prefer the closed platforms to the open platforms. After quickly equilibrating to the testing arena, less anxious rodents will explore and spend increasing time on the open platforms. The LD box consists of two connected chambers, one illuminated while the other is not. The natural preference of the rodent is the dark chamber; however, given time less anxious rodents again will explore and spend increasing time in the light chamber. The OFT is a square testing arena with four walls. In novel settings, rodents prefer to remain unexposed to predators, in this case, close to the wall. After exposure, less anxious animals will cross the arena exploring and spend increasingly more time in

the center. SD paradigms vary to some extent but primarily involve repeated exposure of a rodent to another dominating rodent. The exposed rodents display depressive-like symptoms but also social avoidance. Social avoidance is most commonly measured using SIT. SIT is conducted in a two-chamber arena separated by a wall to prevent contact but allow for other sensory exchanges, i.e., visual cues, odor, and ultrasonic vocalizations. More anxious, socially avoidant rodents will spend less time in the area closest to the neighboring chamber after habituation. These consist of the major testing paradigms used to proximate anxiety in the rodent and have allowed for a more comprehensive understanding of the neuroepigenetic regulation of anxiolytic behavior.

Natural variation in susceptibility to clinical anxiety has been subject to increased scrutiny in recent years. Early animal work suggested that gene-environment interactions likely mediated anxiety outcomes as SD paradigms among other stressors produced anxious phenotypes. Notably, an early study showed that susceptibility to SD, as measured by reduced interaction time in the SIT, was correlated with DNA methylation of CpG islands in the promoter of the *CRH* gene in paraventricular nucleus (PVN) [32]. Natural variation in maternal care during the first week of life was shown to differentially pattern the methylation of nr3c1 promoter of offspring, modification that persisted into adulthood and corresponded to reduced glucocorticoid receptor expression and enhanced HPA axis activation to an acute stressor [33]. These offspring were later characterized as displaying differential anxiety-like behaviors as a consequence of maternal care received as measured by the EPM and OFT [34, 35]. In adult mice, voluntary exercise has been demonstrated to increase nr3c1 expression while reducing miRNA-124, known to inhibit nr3c1, expression [36]. Though in contradiction to other findings, voluntary exercise decreased time in the open arms of the EPM suggesting an increasingly anxious phenotype. Recently, long noncoding RNA (lncRNA) expression of gomafu in the prefrontal cortex (PFC) has been shown to regulate time spent in the center of the OFT and grooming time to suggest that expression of this lncRNA is necessary for reducing anxiety-like behaviors [37]. Likewise, loss-of-function l3mbtl1, null mice show reduced latency to enter the light chamber in the LD box and increased time spent in the center of the OFT [38]. As l3mbtl1 codes for a methylated lysine domain histone-binding protein, a so-called chromatin reader, this suggests that histone lysine methylation is required for regulating anxiety-like behavior. In this vein, tlr4 null mice did not show increased synaptic enrichment of NR1 following in the short term following repeated ethanol exposure nor increased GluR1 enrichment in the long term in the mPFC compared to similarly treated wild-type controls. These tlr4 mice failed to show mPFC enrichment of acetylated-H4 at the promoter of fosB and BDNF in response to ethanol exposure. This observation suggests that tlr4 is necessary for histone H4 acetylation at fosB and BDNF following ethanol exposure and appears to be necessary for ethanol-induced increases in anxiety-like behavior as indicated by time spent in the open arms of the EPM [39]. In contrast, others have shown that acute ethanol exposure reduces amygdalar miRNA-494 subsequently increasing Cited2, CBP, and p300 expression. These changes were associated with increased H3 acetylation in the central amygdala and anxiolysis [40].

8.6 Transgenerational Epigenetics

Transgenerational epigenetic can be either direct inheritance of mRNAs, protein, or DNA modification via the germline or indirect "inheritance" such that the feed-forward phenotypic profile of the parent can lead to changes in either noncoding RNA expression, histone modification, or DNA methylation. Indirect inheritance was shown by Weaver et al. (2004) where cross-fostering experiments suggested that maternal care alone determined GR 1-7 promoter methylation in offspring hippocampi [33]. Morgan and Bale (2011), in a case of direct inheritance, showed that prenatal stress can lead to alterations in stress sensitivity and miRNA expression in the brains of male offspring [41]. These effects persist for several generations suggesting direct inheritance of paternal miRNAs or DNA methylation via sperm.

Transgenerational effects have been consistently observed in the offspring of Holocaust survivors [42–44]. Maternal PTSD of these survivors has been predictive of offspring PTSD risk and increased corticosteroid sensitivity. In specific importance to this chapter, offspring of Holocaust survivors were found to be at a far greater risk of developing an anxiety disorder compared to control, age-matched offspring born to Jewish parents [42]. At this date, the number of generations out to which this inheritance persists and affects offspring of survivors remains unknown. Transgenerational non-genomic transmission of both maternal behavior and HPA axis activation in rats was initially demonstrated by Meaney et al. [45]. The same group showed that glucocorticoid sensitivity and anxiety-like behavior are patterned by maternal care and can persist out for several generations [33, 35]. The level of maternal care during the first week of life patterned the methylation of the GR 1-7 promoter and subsequently GR expression in the hippocampus. These phenotypes can be reversed however by cross-fostering offspring of low-licking and grooming dams with high-licking and grooming dams. In anxious adults of low-licking and grooming dams, the phenotype can be reversed by supplication of an HDAC inhibitor to the hippocampus [34, 35]. Conversely, in low-anxiety adults of high-licking and grooming dams, the phenotype can be reversed by infusion of a methyl donor to the hippocampus [35]. Interestingly, maternal care has also been shown to affect peripheral oxytocin receptor (OXTR) methylation status in rats [46]. A recent clinical study also found that peripheral *OXTR* methylation was associated with increased frequency of anxiety and depression [47]. Genome-wide methylation analysis in infants of mothers with depression and/or anxiety revealed a number of CpG islands to be differentially methylated [48]. Similarly, increased methylation of the *BDNF* gene in blood of adults has been linked to lower maternal care and interpersonal violence-related PTSD [49, 50]. In addition, poor maternal care and anxiety has been linked to risk of diabetes and metabolic syndrome in bonnet macaque offspring [51, 52]. In high- and low-anxiety bred rats, increased H3K9me3 accumulation was found at both the GR and *FGF2* promoters in the hippocampus [53]. This group also found differences in DNA methylation of the *FGF2* promoter in the hippocampus between high- and low-anxiety rats. High-anxiety rats had reduced DNA methylation and methyl-binding protein association at the *FGF2* promoter, which presumably was permissive for increased FGF2 expression [53]. This group

also showed that FGF2 increases H3K9me3 association with both the GR promoter and its own. This demonstrates a potential mechanism by which early-life perturbations independent of maternal care can contribute to anxiety-like behavior across generations.

8.7 Neuroepigenetic Effects of Early Stress on Anxious Behaviors

Early-life stress has been demonstrated repeatedly to pattern stress reactivity and anxious behavior. These changes persist beyond the time frame of the initial stressor and often long into adulthood. The prenatal effects of stress lead to dysregulation of the HPA axis associated mainly with changes GR expression [35]. Though these findings were first reported in animal studies. Recently, these findings have been recapitulated in longitudinal human studies. For instance, maternal prenatal anxiety has been shown to predict internalizing and anxiety scores on the child behavior checklist in the infant [49]. Further, differences in global DNA methylation were observed at a number of CpG sites in neonatal cord blood of mothers affected by anxiety during gestation [48]. Likewise, maternal PTSD has been shown to associate with both increased glucocorticoid sensitivity in the offspring of Holocaust survivors and increased offspring diagnosed with anxiety disorders [42]. Maternal PTSD has also been demonstrated to be predictive of offspring PTSD and presumably through inherited stress reactivity [43, 44]. These findings suggest that both the prenatal environment and stress/trauma history may recruit epigenetic processes in the intergenerational transmission of HPA axis dysregulation and anxiogenic consequences. However, consideration of allostatic load must be of concern as severe and mild stress have opposing roles on physiology and behavior. Allostatic load is the cumulative effect of multiple stressors taking into consideration severity, duration, and ability to cope with stressors [54, 55]. Consider the effects of a severe uncontrollable stressor, for example, maternal separation, on stress sensitivity in contrast to a mild controllable stressor such as voluntary exercise. While maternal separation sensitizes the HPA axis of the infant, voluntary exercise can promote resiliency to future stressors [56, 57].

8.7.1 Prenatal Stress

In utero exposure to maternal stress and corticosteroids patterns the HPA axis of infants ultimately altering synaptic connectivity, function, and behavioral responses specifically those involved in stress adaptation [58–60]. Prenatal restraint stress has been shown to impair offspring brain function and development reducing HPA axis feedback and altering neuroplasticity [61]. Prenatal stress and glucocorticoid treatment produce lasting behavioral changes such as spatial learning impairment and increased anxiety-like behavior [58, 59]. In addition, mild stressors, for instance, postnatal handling, have been shown to reduce these deficits as well as attenuate

HPA axis sensitivity [58, 59]. Prenatal stress does so by altering synaptic connectivity, neurogenesis, and chromatin structure in stress-sensitive regions of the brain, for example, in the PFC where offspring of maternally stressed dams show reduced dendritic spine complexity and density [60]. Similarly, in both rodent and nonhuman primate models, prenatal stress retards hippocampal neurogenesis in the dentate gyrus. Prenatal stress has been linked to increased methylation of the GR 1-7 promoter in the hippocampus as well as reduced methylation of the CRF promoter in the hypothalamus and amygdala of male but not female mice [62]. These sex-specific changes have been linked to differential expression of DNA methyltransferase 1 (DNMT1), though the changes responsible for this dichotomized expression remain unknown. Elliot et al. (2010) first ascribed natural variation in social interaction following social defeat in adults to be due in part to the methylation of the CRH promoter in the hypothalamus. Mice susceptible to social defeat show increased social anxiety and reduced CRH promoter methylation in the PVN [32]. The methylation status of the CRF promoter in PVN helps to explain natural variability in the susceptibility of mice to social defeat and consequently social anxiety. Prenatal stress had previously been shown to differentially affect CRF release in the PVN [63]. Interestingly, a subsequent study found that prenatal restraint stress increased both anxious behavior and corticosterone release in response to stress while reducing CRF promoter methylation at the same CpG islands noted by Elliot et al. (2010) [64]. Prenatal restraint stress has also been shown to increase methylation of the *REELIN* promoter in the PFC perhaps linking changes in synaptic connectivity observed there to underlying molecular influences [65]. *REELIN* is an important neuroplasticity gene, known to be epigenetically regulated by fear conditioning [66]. Similarly, prenatal exposure to maternal depression and anxiety has been linked to increased *NR3C1* 1F promoter methylation and increased salivary cortisol following exposure to a stressor in infants [67, 68]. Maternal anxiety has been linked to differential methylation of a number of other genes in cord blood including *IGF2* and *H19* [69]. In fact, distress during pregnancy has been linked to placental methylation of a number of stress-related genes including *HSD11B2*, *NR3C1*, and *FKBP5* [70]. Other perturbations, including maternal diet and paternal exposure to drugs of abuse such as cocaine and ethanol, have been shown to alter cortical gene expression through changes in the epigenetic machinery and affect anxiolytic behavior in the offspring [71–73]. Importantly, mild postnatal stressors have been shown to reverse the effects of prenatal stress as well as promote resiliency [58, 59, 74, 75]. Given the association between maternal stress and anxiety, these findings provide evidence for the efficacy of behavioral therapy and alike as an early-life intervention [7, 76].

8.7.2 Early-Life Stress

The vast majority of studies of early-life stress focus on the epigenetic consequences of the interactions within the mother-infant dyad. Both maternal care and separation have been demonstrated to both alter HPA axis stress reactivity and adult anxiety

behaviors of the infant through lasting changes to the epigenomes [35, 77]. Specifically, maternal separation has been shown to sensitize offspring HPA axis activation early-life interventions including environmental enrichment attenuate this effect [78]. Poor rearing conditions have been shown to increase CRF release from the PVN and amygdala as well as hypermethylate the GR 1-7 promoter in the hippocampus [33, 79]. Conversely, good maternal care and rearing conditions have been demonstrated to hypomethylate the GR 1-7 promoter in the hippocampus, produce efficient stress responses, and reduce anxiety-like behaviors [35, 80–82]. The GR 1F promoter is the human ortholog of the rodent 1-7 promoter [83]. Hypermethylation of the 1F promoter in the brains of suicide victims was associated with childhood abuse [84]. The findings of McGowan et al. (2009) were later expanded to include the 1-B, 1-C, and 1-H promoters as well [85]. Other groups have failed to replicate some of these findings, however [86]. The McGowan group has also shown that hippocampal ribosomal RNA expression is reduced in suicide victims suggesting reduced hippocampal protein synthesis [87]. Childhood adversity has also been linked to increased 1F promoter methylation in peripheral cells as well [88, 89]. Methylation patterns as a consequence of childhood abuse overwhelmingly persist into adulthood [90]. Early postnatal stress followed by subsequent adult chronic stress has been linked to reduced hippocampal plasticity and increased anxiety-like behaviors [91]. Maternal separation has been shown to reduce amygdalar neurotensin receptor 1 (NTSR1) expression through increased methylation of the *NTSR1* promoter. Microinfusion of NTSR1 receptor agonist increased conditioned freezing responses, while an agonist reduced this behavior suggesting an epigenetic molecular mechanism sufficient for increasing anxiety-like behavior [92]. Similarly, maternal separation has been linked to increased HPA activation to environmental stressors in adult offspring [93]. More recently, however, this finding was both replicated and associated with hypomethylation of the POMC, the gene encoding the precursor for ACTH, in the pituitary [94]. As HPA axis dysregulation has been associated with anxiety-like outcomes, again these findings suggest a critical role of these molecular influences as a consequence of stress in the context of anxiety outcomes. Clinical work has recently shown that early childhood trauma affects CpG methylation in both the promoter and gene proper of the 5-HT3Ar in blood [95]. Interestingly, this locus is downstream of GR response element which showed altered CpG methylation associated with emotional neglect and CpG methylation associated with anxiety-related behaviors.

Adolescence represents another postnatal life stage sensitive to the epigenetic effects of stress [96–100]. For instance, chronic variable stress during adolescence reduces hippocampal volume and spatial cognition, these effects persisting into adulthood [101]. Isolation rearing in adolescent mice reduces the expression of 5-α-reductase I, the rate-limiting enzyme for allopregnanolone, a hormone shown to reduce depressive- and anxiety-like symptoms in rodents [102, 103]. Isolated juveniles show increased CpG methylation upstream of the transcription start site of the *SRD5A1* gene, which codes for this enzyme; one of these islands was demonstrated to be sufficient to reduce expression in the PFC [102]. In adolescent rhesus monkeys, anxious temperament is associated with increased methylation

and reduced expression of the *BCL11A* and *JAG1* genes, associated with neuroplasticity, in the amygdala [104]. Similarly, these findings have been supported by recent clinical work identifying a correlation between *NR3C1*, the gene coding for the glucocorticoid receptor, 1F promoter methylation in blood, and internalizing symptoms [105]. Moreover, these adolescents showing increased 1F promoter methylation and displaying internalizing behavior also had higher concentrations of cortisol upon waking. These findings, in tandem, indicate a significant role of neuroplasticity and HPA axis regulation in stress-sensitive regions of the brain, notably the hippocampus, amygdala, PVN, and PFC, during adolescence and may underscore potential individual variations that contribute to anxious susceptibility. These epigenetic predispositions may be compounded by other environmental perturbations such as exposure to drugs of abuse. Intermittent alcohol exposure, for instance, has been shown to increase HDAC activity in the rodent amygdala [106]. These changes were also associated with reduced time spent in the open arms of the EPM and in the light compartment of the dark/light box into adulthood. Further alcohol-exposed adults had reductions in the number of spines and increased alcohol intake. Conversely, acute alcohol exposure during adolescence produces similar changes in anxiety-like behaviors while decreasing HDAC activity in the rodent amygdala [107]. In summation both predisposition and environmental perturbation may work in synchrony during adolescence to dysregulate both transcription and synaptic integrity in the amygdala and ultimately help shape entrain anxious behavior.

8.8 Stress in Adulthood

Stress induces lasting changes in heterochromatin structure, ultimately changing neuronal plasticity and behavior. The hippocampus, PFC, and amygdala are targets of glucocorticoids. As these regions help regulate spatial memory, executive function, and fear responses, respectively, they are of the utmost importance in the context of anxiety. These regions are extremely sensitive to both acute and chronic stressors and express a large number of epigenetic enzymes and display profound structural changes at the synaptic level in response to environmental stressors. Stressors often produce some type of learning, the spatial and contextual components of which are presumed to be coded by the hippocampus and the cue-based components coded by the amygdalar [24, 108]. The reconsolidation and extinction of these associations are mediated by the PFC. Dysregulation of these memories may fail to attenuate improper responses to environmental stimuli, much like the symptoms of anxiety. Fear conditioning is widely used to study learning and neuroplastic consequences thereof as well as to model symptoms of a number of anxiety disorders as well as other stress-related disorders such as post-traumatic stress disorder [109, 110]. Epigenetics has been thought to be a potential basis of memory on the molecular level [111–113]. Initially, Sweatt et al. (2004) first demonstrated the role of hippocampal histone acetylation during fear memory formation [114]. Miller and Sweatt (2007) later showed that fear conditioning upregulated expression of

hippocampal DNMT3A and 3B, and that DNMT activity there was required for fear memory consolidation [66]. Hippocampal methylation of reelin, PP1, and BDNF was also changed by fear conditioning [115]. Interestingly, reelin and BDNF have well-established roles in dendritic remodeling, and PP1 codes for a phosphatase that acts at histone H3S10 [116, 117]. Presumably these are the grounds for its role in memory as the dual acetylation-phosphorylation H3 mark was enriched at the *BDNF* locus in the hippocampus. Others have found similarly that both histone modification and DNA methylation play critical roles in the amygdala in memory reconsolidation and consolidation, respectively [118]. Tsai et al. (2007) established that both environmental enrichment and HDAC inhibition were sufficient for restoring deficits in memory and synaptic connectivity in a mouse model of neuronal cell loss [119]. A later study by the same group (2009) identified HDAC2 to be necessary for the negative impacts on memory [120]. Recall of recent memories results HDAC2 dissociation from the chromatin, which causes increases in H3 acetylation and increased expression of immediate early genes [121]. Recall of less recent memories do not produce such profound changes in HDAC activity. Yet, HDAC inhibition during reconsolidation of remote fear memories allows for H3 acetylation, increased immediate early gene expression, and neuroplastic changes [121]. This suggests that epigenetic control of chromatin structure regulates neuroplastic changes underpinning behavioral outputs related to fear memories.

Social defeat represents another type of stress-based learning producing an anxious phenotype in the defeated. Social defeat is a well-characterized animal model of a number of psychiatric disorders including modeling symptoms of depression and anxiety [122]. The Nestler group was early in demonstrating that social defeat affects hippocampal chromatin signatures [123]. They showed that chronic social defeat increased H3K27me3 repression of the *BDNF* promoter in the hippocampus. Also, the accumulation of this repressive mark was mitigated by antidepressant treatment, inhibiting HDAC2, resulting in increases in H3 acetylation and H3K4 methylation, both marks promoting transcription [123, 124]. The same group also showed that chronic stress or cocaine exposure altered HDAC activity in the nucleus accumbens [125]. DNMT3A expression increases as a consequence of chronic defeat and decreases as a consequence of chronic cocaine which were associated with synaptic changes as well in the same nuclei [126]. Interestingly, natural variation to susceptibility to social defeat has been associated with distinct methylation signatures of the CRF promoter in the PVN [32]. Resilient animals also show increased H3K9me3 and K3K27me3 in the nucleus accumbens [127, 128]. The levels of accumbal H3K9me3 also change in response to cocaine exposure as well as dendritic morphology [129]. Acute stress and chronic antidepressant treatment have also been shown to increase H3K9me3 levels in the hippocampus [130]. This repressive mark appears to accumulate selectively at repetitive elements, specifically retrotransposons (for review see Lapp & Hunter, 2016) in the genome [131, 132]. Interestingly, Alu and LINE1 retrotransposons appear upregulated in PTSD veterans compared to combat deployed controls [133]. Socially defeated animals also show increased basal corticosterone in circulation, reduced time spent in open arms of the EPM and in the light component of the light/dark box, as well as reduced

hippocampal H3 acetylation and increased HDAC5 expression [134]. These deficits, however, were rescued by a moderate, involuntary exercise regiment, a mild stressor [134]. Voluntary exercise, a mild and controllable stressor, alone has been shown to have anxiolytic effects in addition to reducing hippocampal expression of the histone H2 variant H2A.z and increasing expression of mitochondrial-related genes *TFAM* and *NDUFA6* in the same region [135]. These recent findings hark back to the importance of allostasis and suggest an epigenetic underpinning of anxious behaviors. Further, it has been suggested that stress opens up "windows of epigenetic plasticity" that are unique to the stressor and elicit dynamic effects based on previous stress history [136, 137]. The recent work of the McEwen laboratory has provided strong evidence for this nuanced view of the epigenetic effects of stress. While chronic restraint stress resulted in reduced time spend in the light component of the light/dark box, only a novel acute stressor led to persistent reduction in time spend in the light compartment. These differences corresponded to changes in hippocampal long-term potentiation and NMDA receptor expression [136, 137]. Acute restraint stress exposure has also been shown to convert DNA methylation through the addition of a hydroxyl group of *NR3C1* promoter in the hippocampus [138]. More recently, hyper-hydroxymethylation has been observed in regions associated with neuronal plasticity following acute restraint stress in the hippocampus [139].

8.9 Prospects for an Epigenetic Pharmacology of Anxiety

Epigenetic interventions have proven effective in animal models of anxiety and stress, and some psychiatric drugs, such as the mood stabilizer valproate, have known epigenetic effects (valproate is an HDAC inhibitor). Thus, it would appear that the prospects for epigenetic therapies for anxiety disorders are fairly high.

Most pharmacologic studies of drugs with epigenetic activities have focused on histone acetylation, with the HDACs being the major targets. In fear extinction models, which have substantial relevance to human AD, a variety of HDAC inhibitors have been shown to be effective in enhancing extinction [140]. Similarly, HDAC inhibition reversed the group differences in maternal behavior and adult stress reactivity observed by Weaver in his landmark paper on epigenetic programming of maternal behavior [33]. Similarly, the same phenotype and associated anxious behavior could be reversed with central infusion of the methyl donor S-adenosyl-methionine (SAMe) in adult animals [34, 35]. A number of studies have found SAMe to be more effective than placebo in the treatment of depression, though other well-designed trials have had negative results [141, 142]. A recent Cochrane collaboration review concluded that there was not strong evidence for the efficacy of SAMe in depression but that further research was warranted [143]. Studies of the efficacy of SAMe in the treatment of anxiety symptoms, however, are very limited to date. DNA methyltransferase inhibitors such as zebularine, *N*-phthaloyl-L-tryptophan and 5-aza-deoxycytidine have been shown to interfere with fear memory formation in preclinical models [66, 118]. To date, little clinical

work has been done with this class of drugs, likely due to concerns about side effects, which are significant for some of these agents.

The study of epigenetic drug targets for anxiety remains in its infancy, and many questions remain to be adequately researched. One such question is whether these agents actually offer superior outcomes to existing treatments. Another is whether they might be used in combination with both other drugs and behavioral interventions to additive or even synergistic effect. Nonetheless, molecular epigenetics offers a novel class of potential drug targets for disorders like AD which have historically had relatively few molecular mechanisms with which to work.

Conclusions

Epigenetic mechanisms play a clear mechanistic role in animal models of anxiety, and human epigenetic studies suggest that these observations are generalizable to clinical populations. Indeed, some effort has already been made to translate the preclinical findings in the field into the clinic. Nonetheless, significant questions, particularly those relating to the time course and nature of epigenetic changes in humans, remain to be answered. Beyond the borders of what might now be regarded as "classical" epigenetics, novel molecular mechanisms of epigenomic, genomic, and epitranscriptomic plasticity are being revealed in the brain in behavioral contexts relevant to anxiety disorders. Transposons, which are mobile elements of the genome, have been shown to be regulated by stress exposure in both humans and animal models [131, 144, 145]. The mitochondria, which contains its own genome, shows transcriptional regulation in response to stress, and its function in the nucleus accumbens has been linked to anxiety phenotypes and social subordination in mice [146–148]. Even more intriguingly, covalent modification of RNA in the prefrontal cortex, the methylation of adenosine, has been shown to associate with the development of fear memory in mice [149]. This epitranscriptomic effect points to yet another layer of molecular complexity that will need to be incorporated into our models of anxiety, both normal and pathologic in model systems and in the clinic.

While neuroepigenetics is a relatively young science, it is already clear that it has relevance to our understanding of AD. Indeed, it has begun to produce usable translational findings for the treatment of disorders, like depression, which are highly comorbid with numerous anxiety disorders. There is ample reason to believe that neuroepigenetic mechanisms will continue to be a fruitful area of research into the biology of anxiety and AD.

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