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Don't worry; be informed about the epigenetics of anxiety

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Abstract

Epigenetic processes regulate gene expression independent of the DNA sequence and are increasingly being investigated as contributors to the development of behavioral disorders. Environmental insults, such as stress, diet, or toxin exposure, can affect epigenetic mechanisms, including chromatin remodeling, DNA methylation, and non-coding RNAs that, in turn, alter the organism's phenotype. In this review, we examine the literature, derived at both the preclinical (animal) and clinical (human) levels, on epigenetic alterations associated with anxiety disorders. Using animal models of anxiety, researchers have identified epigenetic changes in several limbic and cortical brain regions known to be involved in stress and emotion responses. Environmental manipulations have been imposed prior to conception, during prenatal or early postnatal periods, and at juvenile and adult ages. Time of perturbation differentially affects the epigenome and many changes are brain region-specific. Although some sex-dependent effects are reported in animal studies, more research employing both sexes is needed particularly given that females exhibit a disproportionate number of anxiety disorders. The human literature is in its infancy but does reveal some epigenetic associations with anxiety behaviors and disorders. In particular, effects in monoaminergic systems are seen in line with evidence from etiological and treatment research. Further, there is evidence that epigenetic changes may be inherited to affect subsequent generations. We speculate on how epigenetic processes may interact with genetic contributions to inform prevention and treatment strategies for those who are at risk for or have anxiety disorders.

Keywords

DNA methylation; chromatin remodeling; non-coding RNAs; stress; HPA axis

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1. Anxiety Disorders

Anxiety disorders are characterized by excessive worry and avoidance that cause significant impairment across multiple domains of an individual's life (e.g., school, home, work; American Psychiatric Association, 2013). Children and young adults (ages 10–25 years old) are at highest risk for developing anxiety disorders and almost one third of the child and adult population meets criteria for an anxiety disorder (28.8%; Michael *et al.*, 2007). Although animal models are unable to capture cognitive processes related to anxiety (i.e., worry), studies have examined various behaviors as proxies for anxiety (e.g., decreased interest in social engagement, increased hiding in dark spaces, decreased time to feed). These animal studies, and recent human studies, are beginning to provide insight to the epigenetic mechanisms underlying the emergence and maintenance of anxiety.

The current diagnostic classification system, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5; American Psychiatric Association, 2013), outlines criteria for eleven anxiety disorders including panic disorder and social anxiety disorder, that are described later in this review. In DSM-5, panic disorder is described as an abrupt surge in physiological (e.g., sweating, shaking, chest pain) or cognitive (e.g., fear of losing control or going “crazy”) symptoms. Social anxiety is described as marked fear or anxiety about one or more social situations in which the individual is exposed to possible scrutiny by others. Notably, the human studies described herein only examine panic and social anxiety disorders – other human studies examine “anxiety symptoms” or sub-diagnostic anxiety symptoms. Further, no studies included in the current review examine the high comorbidity of anxiety with other disorders, particularly with depression, that is estimated to be present in more than half (57%) of individuals with an anxiety disorder (Zimmerman *et al.*, 2014). Current treatments (e.g., cognitive-behavioral therapy) for anxiety appear effective in the short-term; however, they have yet to prove efficacious in maintaining these short-term therapeutic gains (Piacentini *et al.*, 2014). The efficacy of pharmacotherapy (e.g., barbiturates and benzodiazepines) is complicated by these high comorbidity rates. As such, better biological insight is needed into the epigenetic processes that underlie the development of anxiety disorders. To this end, several investigations have shed light on epigenetic mechanisms that govern the regulation of genes that contribute to anxiety states. These investigations vary widely in research design, including differences across experimental manipulations, age, brain regions, and genes of interest (Table 1). As a result, we attempted to synthesize the literature according to overlapping features whenever possible. Thus, the review of the animal (preclinical) literature on epigenetics and anxiety is organized by developmental period and brain region. Then, human studies are discussed.

2. Neural Correlates of Anxiety

Animal models have assessed anxiety (Table 2) and associated epigenetic modifications in several brain regions. Not surprisingly, significant attention focuses on structures that regulate the stress response such as the hippocampus, amygdala, hypothalamus, and the pituitary. Striatal regions, such as the caudate-putamen and nucleus accumbens (NAc) known for regulating reward-related behaviors, emotional states and stress responses, have been investigated as well. Additionally, epigenetic alterations within cortical areas that

mediate higher cognitive functions and are important in anxiety are beginning to be examined.

The neuroendocrine system is an important regulator of the stress response. Corticotropin-releasing hormone (CRH, also known as corticotropin-releasing factor or CRF) is released from the paraventricular nucleus (PVN) of the hypothalamus into the primary capillary plexus of the hypothalamo-hypophyseal portal system to stimulate the anterior pituitary to synthesize proopiomelanocortin (POMC) and release adrenocorticotrophic hormone (ACTH), a peptide derived from POMC, into the blood. ACTH in the blood then activates the synthesis and release of corticosterone in rodents and cortisol in humans from the adrenal glands of the kidneys. Cortisol, or corticosterone, then feeds back on the hypothalamus and pituitary as well as the hippocampus to shut down the hypothalamic–pituitary–adrenal (HPA) axis. Dysregulation of this neurochemical system contributes to anxiety.

The contribution of genetic variance to develop an anxiety disorder is estimated to range from 30–50% (Hettema *et al.*, 2001, Smoller *et al.*, 2009) implying that 50–70% of the variance may be due to the environment. The role of environmental factors in the development of anxiety disorders may act through epigenetic mechanisms. Such mechanisms may begin *in utero*, as evidenced by the fact that non-medicated anxiety in the mother associates with altered DNA methylation of the glucocorticoid receptor gene (NR3C1) promoter region in cord blood and in the genome (Hompes *et al.*, 2013, Non *et al.*, 2014). Thus, it is important to consider how changes in the epigenome may contribute to susceptibility of an anxiety disorder.

3. Epigenetic processes

Recent evidence highlights the scientific utility of examining the intersection of genetics and developmental biology – epigenetics. Epigenetics was first described in the mid-twentieth century by Conrad Waddington. He derived the term “epigenetics” from the Greek word “epigenesis” and broadly defined it as: “all those events which lead to the unfolding of the genetic program for development” (Waddington, 1939). This definition has been updated to specify processes that include reversible alterations in gene expression that do not coincide with alterations in DNA sequence and can be transferred to successive mitotic generation of cells (Maze and Nestler, 2011). Further, these epigenetic processes are considered “molecular factors or processes around DNA that regulate genome activity independent of DNA sequence and that are mitotically stable” (Skinner *et al.*, 2010). Herein, we use this definition of epigenetics to interpret and understand findings in the literature on anxiety conducted in humans and animals. The primary goal of this review is to determine the current state of this literature as well as to highlight areas for further study that may improve prevention and treatment of anxiety disorders.

Several related epigenetic mechanisms regulate gene expression: chromatin remodeling, DNA methylation, and non-coding RNAs. These mechanisms may promote epigenetic processes due to environmental impact or genomic imprinting. For example, the expression of parent-of-origin imprinted genes, such as *paternally expressed gene 3* (*Peg3*), are mediated by differential DNA methylation in a locus-specific manner (Reik *et al.*, 1987).

Furthermore, DNA methylation could be transferred to future generations of cells via mitosis and may be succeeded by chromatin remodeling. Alterations of DNA methylation change rapidly, occurring within 1 h and reversing by 24 h (Miller and Sweatt, 2007) or are stable, reflecting events from prior decades (Heijmans *et al.*, 2008). Over the life course of an organism, these changes can accrue and alter its genetic profile (Fraga *et al.*, 2005). Similar to the transfer of DNA methylation to the next generation of cells, non-coding micro-RNAs are hypothesized to be passed onto the next generation in sperm (Murashov *et al.*, 2015).

3.1. Chromatin Remodeling

Chromatin is composed of DNA complexed with proteins. DNA is tightly packaged around a histone core composed of eight histone molecules, two each of histone H3, H4, H2A, and H2B, within a nucleosome, the unit of chromatin. The amino terminal (or histone) tails project from the histone core and are the sites of posttranslational modifications. These tails are accessible to covalent modifications, such as acetylation, methylation, and phosphorylation (Kouzarides, 2007) and determine if the chromatin is in an “open” (activated) or a “closed” (repressive transcriptional) state (Kornberg and Lorch, 1999) or in a state between these two extremes. Because acetylation reduces the electrostatic attraction between the histones and the negatively charged DNA, it creates a more “open” chromatin state (Gardner *et al.*, 2011, Jenuwein and Allis, 2001). Then, chromatin is more loosely packaged and more accessible to the transcriptional apparatus, thereby allowing increased gene expression. For example, acetylation of histone H3, demonstrated in active chromatin (euchromatin) (McQuown and Wood, 2010), appears to boost transcription. Histone acetyltransferases increase acetylation and histone deacetylases (HDACs) maintain it (Jenuwein and Allis, 2001).

Histone methylation is a more complex histone modification system that, depending on the location and number of methyl groups conjugated, may repress or activate gene expression (Barski *et al.*, 2007). Methylation of histones is controlled by both histone methyltransferases (HMTs) and histone demethylases (HDMs). Modifications such as H3K27me3 are found in inactive chromatin while H3K4me3 is found in transcriptionally active chromatin. Phosphorylated histones are found in euchromatin, as well as in inactive chromatin (Ito, 2007). Other modifications such as histone sumoylation, poly-ADP-ribosylation, and ubiquitylation are involved in the regulation of gene expression (Kouzarides, 2007). Taken together, these form a “histone code” that, in part, governs gene expression (Jenuwein and Allis, 2001).

3.2. DNA Methylation

DNA methylation is another primary epigenetic mechanism controlling gene expression marking genes for silencing or activation. Specifically, DNA methyltransferases mark DNA by transferring a methyl group to the 5' position of the cytosine pyrimidine ring to yield 5'-methylcytosine (5'-mC). This occurs often at cytosine:guanine (CpG) dinucleotides to form 5'-methylcytosine guanine dinucleotides (mCG) (e.g., reviewed in Bestor, 2000, Bird and Macleod, 2004, Fazzari and Greally, 2004, Lande-Diner *et al.*, 2004, Robertson and Wolffe, 2000) but non-CpG cytosine DNA methylation (mCH, where H = A, C, or T) has been

identified as well (Varley et al, 2013, Xie et. al., 2012). In adult mouse dentate neurons, approximately three-quarters of DNA methylation occurs at CpG sites (Guo, 2014) and most mCH (non-CpG) sites are mCA (A = adenosine) (Varley et al., 2013, Xie et al. 2012).

Regions of DNA that contain a high density of CpG dinucleotides are “CpG islands” and most are located in promoter regions (Larsen *et al.*, 1992). About 70% of CpG sites in CpG islands are methylated with less than 2% completely unmethylated (Ziller *et al.*, 2013). Methylation of CpGs near transcription start sites diminish gene transcription (e.g., Bird and Macleod, 2004, Campanero *et al.*, 2000, Heller *et al.*, 2008, Hwang *et al.*, 2007, Iguchi-Ariga and Schaffner, 1989, Jaenisch and Bird, 2003, Lande-Diner *et al.*, 2004, Robertson and Wolffe, 2000, Tong *et al.*, 2010, Zardo *et al.*, 2005). For example, it may reduce the binding affinity of cognate transcription factors as is shown for Sp1 and CREB. In addition, DNA methylation may recruit histone modifying enzymes to affect chromatin. Normal development and other epigenetic processes (e.g., X chromosome inactivation and genomic imprinting) depend upon proper DNA methylation (Heller *et al.*, 2008, Robertson and Wolffe, 2000, Suzuki and Bird, 2008, Zardo *et al.*, 2005).

The methylated DNA binding protein, MeCP2, binds DNA at methylated cytosines, and inhibits transcription (Boyes and Bird, 1991, Cross *et al.*, 1997, Gabel et al., 2015, Hendrich and Bird, 1998, Prokhortchouk *et al.*, 2001). MeCP2 may help recruit histone deacetylases to deacetylate nearby histones, resulting in attenuated gene expression (Jones *et al.*, 1998, Nan *et al.*, 1998, Razin, 1998). However, MeCP2 may play a role in recruiting transcription factors, as seen with CREB in active promoters (Chahrour *et al.*, 2008). The DNA methyltransferases DNMT3A and 3B perform *de novo* DNA methylation, while DNMT1 converts hemimethylated DNA formed following DNA replication to the fully methylated state (Bestor, 2000). Post-mitotic neurons of the brain have high levels of DNMT1 while oligodendrocytes and astrocytes have low DNMT1 content (Inano *et al.*, 2000, Veldic *et al.*, 2004). Neuronal maturation and normal development of the brain requires DNMT1, as a conditional knockout of *Dnmt1* inhibits these processes (Fan *et al.*, 2001).

3.3. Non-coding RNA

Non-coding RNAs (ncRNAs) are important for epigenetic regulation of gene expression, specifically in transcriptional and post-transcriptional regulation (reviewed in Mercer *et al.*, 2009). MicroRNAs (miRNAs or miRs), a specific type of ncRNA, are small, 22 nucleotide ncRNAs that are derived from primary miRNAs. Almost two thousand miRNAs are encoded in the human genome (www.miRBase.org) and may target over 60% of the coding genes (Friedman *et al.*, 2009, Lewis *et al.*, 2005). miRNAs expressed in brain may relate to learning and memory (Bredy *et al.*, 2011), synaptic plasticity (e.g., miR-132 and miR-134 in dendrite morphology) (Schratt *et al.*, 2006, Vo *et al.*, 2005), and neuronal differentiation (miR-124) (Makeyev *et al.*, 2007, Visvanathan *et al.*, 2007). This is an emerging area of research in behavioral studies. At this point, there is little research related to anxiety disorders but this will likely expand in the near future.

4. Preconception and Prenatal Manipulations in Animal Studies

Most studies in which manipulations took place during the prenatal or preconception periods utilized parental and/or offspring exposure to nutritional deficiencies, stress, or to drugs of abuse (Table 3). Firstly, effects of altered maternal diet on neural changes and anxiety-like behaviors in the offspring will be examined. Next, we discuss studies of restraint stress in pregnant dams. This method alters the prenatal environment and induces long-lasting changes to the HPA axis in offspring (Kosten *et al.*, 2014b). Lastly, we discuss studies in which sires (fathers) are exposed to drugs of abuse prior to conception resulting in anxiety behaviors and changes to epigenetic machinery in their offspring. In each section below, we review findings that have used the above protocols to alter both an animal's anxiety level and its epigenetic profile.

4.1. Hypothalamus

Maternal stress during pregnancy can disturb neuroendocrine levels and have a negative impact on offspring development. Dams exposed to restraint stress during gestational days 8–21 have adolescent offspring of both sexes with altered *Crh* DNA methylation in the hypothalamus and increased anxiety-like behavior (Xu *et al.*, 2014). Specifically, offspring show decreased entries into the center zone of the open field and decreased entries into the open arm of the EPM. Interestingly, these behaviors are significantly more elevated in male offspring of prenatally restrained dams compared to female offspring. Prenatally stressed offspring exhibit decreased DNA methylation of three CpG sites in the *Crh* promoter region within the hypothalamus and have higher serum corticosterone levels basally and after acute stress. The decreased *Crh* DNA methylation seen in prenatally stressed animals may lead to elevated basal corticosterone levels that become more elevated after stress. Or, maternal stress-induced elevations in basal corticosterone may cause hyper-responsivity of the HPA axis in adolescent offspring. Interestingly, prenatal stress exposure has sex-dependent effects in anxiety-like behaviors but not in *Crh* DNA methylation in hypothalamus.

4.2. Cortex

The quality of nutrition during brain development can alter epigenetic machinery. Dams were exposed to a high or low omega-3 fatty acid diet throughout gestation and lactation (Tyagi *et al.*, 2015). After weaning, the offspring either continued on the same diet or were switched to a western diet until adulthood. Offspring that transitioned from low omega-3 to the western diet displayed more anxiety-like behavior as indicated by decreased time spent in the open arms of the EPM compared to offspring raised and continued on the low omega-3 diet. Interestingly, animals raised on the high omega-3 diet and switched to a western diet did not display changes in anxiety behavior, a finding that suggests that early life exposure to a high omega-3 diet had a protective effect on western diet-induced anxiety. Animals raised and maintained on the low omega-3 diet had increased DNA methylation of the *brain-derived neurotrophic factor (Bdnf)* exon IV promoter within the frontal cortices that was further elevated in low omega-3 animals that transitioned to a western diet. Conversely, animals raised and maintained on the high omega-3 offspring had less DNA methylation of the *Bdnf* exon IV promoter at baseline and this hypomethylation was not observed in the in high omega-3 offspring that had transitioned to a western diet. Overall,

low omega-3 offspring had lower *Bdnf* mRNA and BDNF protein levels, while high omega-3 offspring displayed elevated *Bdnf* mRNA and BDNF protein levels regardless of the transition to a western diet. Notably, the western diet decreased MeCP2 phosphorylation in the low omega-3 and not in the high omega-3 offspring, a possible mechanism contributing to differential *Bdnf* DNA methylation and gene expression between low and high omega-3 offspring.

Similar to dietary interventions, exposure to maternal stress can induce cortical epigenetic changes. Prenatal restraint stress during the last ten days of pregnancy increases anxiety-like effects on the EPM in adult offspring (Palacios-García *et al.*, 2015, Tyagi *et al.*, 2015) that are accompanied by decreased *Reelin* (*Rln*) mRNA expression and increased DNA methylation of the *Rln* distal promoter region. Reelin is a critical protein that regulates brain development through cytoskeleton modifications. The association between decreased *Rln* expression and increased anxiety-like behavior is consistent with previous human *post-mortem* studies that find down-regulation of *Rln* mRNA in patients with schizophrenia and bipolar disorder with psychosis. Therefore, increased DNA methylation of the *Rln* distal promoter region may contribute to the development of anxiety disorders. Thus, environmental disturbances during pregnancy can influence genes that regulate brain plasticity in cortical areas and may determine susceptibility to develop an anxious phenotype.

Paternal exposure to drugs of abuse alters basal anxiety behaviors in offspring, sometimes in a sex-dependent fashion. For instance, adult sires exposed to cocaine prior to mating with a cocaine naïve female produce male offspring that display greater anxiety-like behavior indicated by increased latency to feed in a novelty-induced hypophagia task (White *et al.*, 2015). No behavioral effects are seen in female offspring. Male, but not female, offspring of cocaine-exposed sires have higher *Bdnf* mRNA levels and increased association of histone 3 acetylation with *Bdnf* promoters in the medial prefrontal cortex (PFC) (Vassoler *et al.*, 2013). This association also is seen in the sperm of cocaine sires. Histone acetylation, associated with increased transcriptional activity, may elevate *Bdnf* expression levels as seen in the male offspring of cocaine sires. In contrast, paternal alcohol exposure results in increased anxiety-like behaviors in female, but not male, offspring (Liang *et al.*, 2014). However, paternal alcohol exposure increases DNA methylation of the *paternally expressed 3* (*Peg3*) gene promoter region in offspring of both sexes, a methylation pattern also found in the sperm of sires. Deficiencies in *Peg3* expression cause fetal growth restriction as well as anxiety-like and abnormal maternal behaviors. However, the possibility that altered maternal behavior towards pups sired by males exposed to drugs of abuse contribute to this heightened anxiety in offspring has not been supported in the paternal cocaine exposure study (Vassoler *et al.*, 2013). Thus, offspring of fathers exposed to environmental disturbances prior to conception may be at risk for developing an anxious phenotype and such effects may be sex-dependent and occur through epigenetic changes in cortical areas.

5. Early Life Manipulations in Animal Studies

The majority of early life studies focus on changes in offspring behavior as a result of manipulations in parental care (Table 4). Maternal separation is a commonly used paradigm

in which a rodent pup is separated from its dam for an extended period of time on multiple occasions. These studies vary in the age of the pup during separation, hours of separation each day, days of separation, etc. (Kosten *et al.*, 2012). Other studies investigate how litter gender composition or injury to the dam alters maternal behaviors, such as pup licking, and whether or not changes to maternal behaviors affect offspring behavior or the epigenome. In other cases, offspring of high-licking/grooming and arched-back nursing (LG-ABN) dams are compared to offspring of low LG-ABN. A model used in nonhuman primates examines effects of variable foraging demand, a task where animals cannot predict whether food will be easily accessible on a given day, also will be reviewed. This task not only strains the dyadic relationship between mothers and offspring but disrupts the stress response in offspring for many years (Coplan *et al.*, 1996). Nonhuman primates with high and low anxious temperament show DNA methylation differences and these data will be reviewed as well. In addition to maternal paradigms in mammals, the effects of paternal deprivation in offspring have been examined in a fish model.

5.1. Hippocampus

Maternal separation can alter the epigenetic machinery of several genes in the hippocampus. A single 24 h episode of maternal separation at PD9 in male C57BL/6J and DBA/2J mice resulted in reduced anxiety-like behaviors evidenced by increased time spent in the center of the open field compared to control animals (Kember *et al.*, 2012). Male mice from both strains showed an increase in *arginine vasopressin* (*Avp*) gene DNA methylation within the promoter region due to maternal separation. Conversely, maternally separated DBA/2J females spent less time in the center of the open field compared to their respective controls. Maternal separation decreased DNA methylation of *brain-expressed nuclear hormone receptor* (*Nr4a1*) in C57/BL6 mice, but not in the DBA/2J strain. Separation increased DNA methylation within the exon 1₇ promoter region of the *glucocorticoid receptor* (*Nr3c1*) in DBA/2J animals, but not in the C57/BL6 strain. In this study, male offspring displayed resilience to maternal separation-induced anxiety possibly as a result of decreased *Avp* expression. Maternal separation also altered *Nr4a1* and *Nr3c1* DNA methylation in a strain-dependent manner, suggesting possible genetic factors influence responses to acute stressors.

DNA methylation of *Nr3c1* also differs between offspring of low and high LG-ABN dams. Adult male offspring of low-LG-ABN mothers show elevated *Nr3c1* DNA methylation within the exon 1₇ promoter region compared to offspring of high-LG-ABN mothers (Weaver *et al.*, 2004). CpG site 16, within the *Nr3c1* exon 1₇ promoter region, contains a binding site for the transcription factor nerve growth factor-inducible protein A (NGFI-A). DNA methylation CpG site 16 was consistently elevated in offspring of low-LG-ABN mothers and rarely elevated in offspring of high-LG-ABN mothers. The role of maternal care in these effects was demonstrated in adoption study where offspring of Low- or High-LG-ABN were cross-fostered to either Low- or High-LG-ABN dams within 12 h of birth. High-LG-ABN offspring fostered by Low-LG-ABN dams showed a similar increased DNA methylation at CpG site 16 within the exon 1₇ promoter as Low-LG-ABN biological offspring. Likewise, Low-LG-ABN offspring fostered by High-LG-ABN dams showed decreased DNA methylation at this region indistinguishable from High-LG-ABN biological offspring. Adult offspring of High-LG-ABN also exhibited greater H3K9 acetylation and

greater binding of the NGF1-A transcription factor with the exon 17 promoter region. Relatedly, another study found that High-LG female offspring were less anxious; they spent proportionally more time in the center of an open field (Pan *et al.*, 2014). They also displayed elevated DNA methylation levels at CpG sites 7 and 17 of the *Nr3c1* exon 17 promoter. These studies suggest that high levels of maternal care associate with low anxiety behaviors and with sex-dependent DNA methylation effects at the glucocorticoid receptor gene.

Maternal behavior in rodents is heavily influenced by the sex of the pup and is altered by modifying litter gender composition (LGC) in which offspring of single-sex litters are compared to offspring of mixed-sex litters. We found that males from single-sex litters spent the most amount of time while females from single-sex litters spent the least amount of time in closed arms of the EPM (Kosten *et al.*, 2014a). *Nr3c1* DNA methylation was higher at two CpG sites for females regardless of LGC, whereas *Bdnf* DNA methylation levels tended to be higher in single-sex litter male rats compared to mixed-sex litter male rats. Lastly, we found increased DNA methylation of the mu-opioid receptor (*Oprm1*) gene in single-sex litters compared to mixed-sex litters (Hao *et al.*, 2011). Higher hippocampal *Bdnf* DNA methylation in single-sex males may have contributed to decreased BDNF levels that, in turn, facilitated elevated anxiety levels. Overall, results suggest that the hippocampus is one brain region in which early life experiences can alter epigenetic processes and associated behaviors often in a sex-dependent or strain-dependent manner.

5.2. Amygdala

Maternal separation alters genes in the amygdala that regulate the activity of neurotransmitters. Adult male rats with 3 h/day of maternal separation from PD 2–14 displayed increased DNA methylation of the *neurotensin receptor 1* (*Ntsr1*) promoter region. The product of this gene, neurotensin receptor 1, is a G-protein coupled receptor that mediates the function of neurotensin, an endogenous neuromodulator of dopamine transmission (Toda *et al.*, 2014). Although this study did not find any effect of maternal separation in EPM or open field, conditioned fear was enhanced. Another study found that maternal separation from PD 14–21 increased anxiety-like behavior as seen by the decreased percentage of open arm entries and reduced time spent in the open arms of the EPM when tested at PD21 (Park *et al.*, 2014). Separated animals displayed increased DNA methylation of *synapsin 1* (*Syn1*) around the transcription start site, a finding that compliments the decreased *Syn1* mRNA and Syn1 protein expression in the amygdala of separated animals. Syn1 tethers synaptic vesicles to actin filaments and phosphorylation reduces this binding, to allow vesicles to enter the readily-releasable pool. Because mitogen-activated protein kinases, Map4k1 and Map4k2, phosphorylate Syn1, the maternal separation induced decrease in DNA methylation and increase in mRNA expression of these kinases may have contributed to anxiety-like behavior via amygdalar Syn1-Mapk alterations to synaptic neurotransmission.

Anxiety behaviors also associate with elevated levels of *Dnmt1* and increased DNA methylation of neuroplasticity-related genes. In a rodent model of neuropathic pain, mother rats that suffer from chronic constriction injury (CCI) exhibit defective maternal care. Adult

offspring cared by CCI dams, biological or cross-fostered, show increased anxiety-like behaviors indicated by decreased time in the open arms of the EPM and decreased time in the center of the open field compared to offspring of sham-operated dams (Zhong *et al.*, 2015). Further, these offspring have increased *Dnmt1* expression in the amygdala although no differences are found in *Dnmt3a* and *Dnmt3b* expression. Total DNA methylation is upregulated in the offspring cared for by CCI dams.

Alisch et al (2014) examined the role of DNA methylation in the development and expression of anxious temperament, an important risk factor for the later development of anxiety and depressive disorders. Their findings showed that young male rhesus macaques with high anxious temperament have increased DNA methylation of *BCL11A* and *JAG1* genes, whose products are implicated in neurite arborization and neurogenesis, respectively. Higher levels of *Dnmt1* in the amygdala of anxious animals may have led to increased transcriptional silencing of genes associated with neuroplasticity. Thus, the amygdala is susceptible to epigenetic alterations in a number of genes related to plasticity due to early life experiences.

5.3. Striatum

DNA methylation changes within the striatum are seen with LGC. We found higher DNA methylation levels of *Nr3c1* in the NAc of less anxious female single-sex rats at 3 specific CpG sites (Kosten *et al.*, 2014a). *Early growth response protein 1* (*Egr1*) DNA methylation at the -501 CpG site was lowest in male single-sex rats that displayed greater anxiety-like behaviors. *Egr1* codes for the transcription factor NGFI-A that has a binding site on exon 1₇ of the *Nr3c1* promoter region. *Bdnf* DNA methylation levels did not differ by LGC or sex. Additionally, DNA methylation of the *Oprm1* promoter region tended to be higher in the NAc and caudate-putamen of single-sex males (Hao *et al.*, 2011). Hence, maternal behavior may influence anxiety-like behaviors in single-sex male litters via decreased *Egr1* DNA methylation in combination with increased *Oprm1* DNA methylation in the NAc. Similar to the hippocampus, early life experience can have sex-dependent epigenetic and behavioral effects.

5.4. Hypothalamus and Pituitary

Maternal separation for 3 h/day from PD 1–10 induced DNA hypomethylation of the *Avp* enhancer region in the PVN of the hypothalamus of male offspring at six weeks, three months, and one year of age compared to control mice (Murgatroyd *et al.*, 2009). Female offspring were not examined. Further experiments identified that, under normal conditions, MeCP2 bound to selective regions of an *Avp* enhancer to repress gene expression. Maternal separation triggered MeCP2 phosphorylation, a reaction that reduced MeCP2 binding to the *Avp* enhancer region and contributed to *Avp* DNA hypomethylation. Maternal separation reduced MeCP2 occupancy in six week old mice via DNA hypomethylation of CpG island 3 within the *Avp* enhancer region. Despite these epigenetic alterations, this study reported no differences in male offspring behaviors on the EPM, light-dark box, and novelty-induced hypophagia tasks although basal corticosterone levels were altered. In a separate study, maternal separation for 4 h/day from PD 2–13 decreased *Crh* DNA methylation in two CpGs preceding (CpG1) and inside (CpG2) the cyclic AMP-responsive element (CRE) in the *Crh*

promoter within the PVN but not in the central nucleus of the amygdala in adolescent rats of both sexes (Chen *et al.*, 2012).

POMC, a prohormone from which ACTH is derived, is a key component of the HPA axis. *POMC* synthesis is regulated by two hypothalamic hormones, CRH and AVP, the genes of which are susceptible to stress-induced epigenetic alterations. Maternal separation induced DNA hypomethylation of the distal *Pomc* promoter region accompanied by elevated *Pomc* mRNA levels (Wu *et al.*, 2014). Under normal conditions, MeCP2 recruits Dnmt1 and Hdac2 to repress *Pomc* expression in the mouse pituitary. Maternal separation may reduce MeCP2 binding and diminish DNMT1 recruitment to the *Pomc* promoter region in the pituitary, leading to elevated levels of POMC. These findings suggest that maternal separation may induce long-lasting epigenetic changes to neuroendocrine systems that regulate the HPA axis.

5.6. Prefrontal Cortex & Whole brain

Early life experiences can affect cortical genes associated with neural plasticity. One investigation used a rodent model of maltreatment in which pups were exposed to a stressed-abusive dam or a positive caregiving dam for 30 min/day during the first postnatal week (Roth *et al.*, 2009). Exposure to a neglectful caretaker decreased *Bdnf* mRNA expression in the PFC, accompanied by increased *Bdnf* DNA methylation at multiple CpG sites within the promoter region, in adult animals of both sexes. Further, maltreated females displayed increased prepartum anxiety-related behaviors and deficits in maternal behavior towards their own offspring. Their offspring also had increased *Bdnf* DNA methylation in the PFC, an effect that was not rescued by cross-fostering. This study supports evidence that adverse early life experiences may augment behavior and induce epigenetic changes that may be passed to subsequent generations.

Another study used a fish model to assess the effects of paternal care on the epigenome of offspring (McGhee and Bell, 2014). Stickleback fathers are the sole provider of offspring care. Fertilized eggs were split into two categories; “orphaned”, which were reared without direct care from their biological father, and “father-reared”, which were cared for by their genetic father. Anxiety-like behaviors and *Dnmt3a* expression were compared between father-reared and orphaned siblings. Overall, orphaned offspring displayed heightened anxiety-like behavior in a new environment and during a predator encounter compared to father-reared offspring as indicated by increased amounts of time pecking at the tank walls in these situations. This behavioral difference related to the type of care father-reared offspring received from their fathers. Orphaned offspring deprived of high direct paternal care showed elevated anxiety levels. Conversely, orphaned and father-reared offspring of low paternal care fathers exhibited low anxiety levels. Thus, elevated anxiety levels in offspring were present only when high direct paternal care was deprived as there were no changes to offspring anxiety levels in the absence of low direct paternal care. Furthermore, fathers that provided high paternal care had father-reared offspring with higher *Dnmt3a* expression in their brains compared with orphaned siblings. In contrast, fathers that provided lower direct paternal care had offspring with lower *Dnmt3a* expression compared with orphaned siblings. High paternal care resulting in higher *Dnmt3a* expression may have led to increased DNA

methylation of genes associated with anxiety-like behavior. Additional animal models should further examine the influence of paternal care on offspring development.

5.7. Blood Lymphocytes

A nonhuman primate study found associations between global DNA methylation and reactivity to stress. Young female rhesus macaques that underwent a variable foraging demand, a procedure where mother and daughter dyads could not predict whether or not food would be easily accessible on a given day, displayed higher *serotonin transporter* (*5-HTT* or *Slc6a2*) and global DNA methylation, and greater behavioral reactivity to high intensity stressors, an indicator of heightened anxiety (Kinnally *et al.*, 2011). This study provides evidence that stressful experiences early in life alter global DNA methylation patterns and influence stress responsiveness later in development. That such effects can be measured in lymphocytes affords the opportunity to conduct more research in humans.

6. Adult and Juvenile Manipulations in Animal Studies

Studies in adult and juvenile animals include altering gene expression, identifying differences between selectively bred strains, and assessing effects of exposure to environmental stressors (Table 5). First, studies in which a target gene is knocked-out or overexpressed, sometimes in a neuron-specific manner and codes for a particular protein or miRNA that facilitates a change in animal behavior, will be reviewed. Second, differences in DNA methyltransferase expression between selectively bred rat strains that display differences in novelty exploration and emotional reactivity are discussed. Third, four weeks of corticosterone treatment, beginning in adolescence and ending in early adulthood, is used to mimic effects of elevated cortisol in humans because this associates with the development of many behavioral disorders, including anxiety disorders. Lastly, we will review studies in which a methyl-donor-deficient diet in early life influences DNA methyltransferase gene expression and behavior.

A few studies have used stress paradigms to identify epigenetic changes associated with anxiety-like behaviors. Chronic unpredictable stress (CUS) induces a number of behaviors homologous to symptoms experienced by people with anxiety and mood-related disorders (Willner, 2005). In this protocol, animals are exposed to a series of unpredicted stressors (i.e., 24-hrs of wet bedding, five min cold swim, etc.) once or twice a day, often over the period of a month. Social defeat stress follows the resident/intruder paradigm (Miczek, 1979) and usually involves subjecting a C57/BL6 mouse to a large and physically aggressive CD-1 mouse for ten min a day over ten days. The social defeat paradigm reliably induces depressive- and anxiety-like behaviors in adolescent (Iñiguez *et al.*, 2014) and adult (Berton *et al.*, 2006) mice that can persist for weeks.

6.1. Hippocampus

Gene silencing of an epigenetic repressor alters gene expression in the hippocampus. KRAB-associated protein 1 (*Kap1*, also known as *Trim28*) is highly expressed in the hippocampus and functions as a corepressor with other scaffolding proteins to silence transcriptional activity. Forebrain-specific *Kap1* knockout mice display increased anxiety-

like behaviors evidenced by decreased entries and time spent in the open arms and more time in the closed arms of the EPM (Jakobsson *et al.*, 2008). *Kap1* knockout mice also make fewer entries into and move less in the center of an open field. Interestingly, this anxiety-like profile is accompanied by decreased trimethylation of histone 3 lysine 9 (H3K9me3) and increased H3 and H4 acetylation at the *makorin ring finger protein 3* (*Mkrn3*), and *protocadherin β 6* (*Pcdhb6*) promoter regions. In humans, the *MKRN3* gene is paternally imprinted and disruption of imprinting is implicated in Prader-Willi syndrome. The *PCDHB6* gene encodes a cell adhesion molecule associated with maintaining specific synaptic connections. Therefore, forebrain *Kap1*-deletion may increase transcriptional activity of anxiety-inducing genes, including genes that are normally imprinted, as evidenced by increased H3 and H4 acetylation and decreased levels of the transcriptional repressor H3K9me3 at promoter regions.

Studies comparing selectively bred rat strains that differ in baseline anxiety-like behavior reveal that epigenetic changes within the hippocampus may be age-dependent and subregion specific. These strains include bred low responders (bLR) that exhibit high anxiety-like behavior and bred high responders (bHR) that display low anxiety levels. Male animals were assessed for *Dnmt1* expression at PD 7, 14, and 21. At baseline, anxiety-prone bLR rats exhibited lower *Dnmt1* mRNA expression in the upper dentate gyrus and the CA3 region of the hippocampus as early as PD 7 compared to bHR rats (Simmons *et al.*, 2013). Conversely, bLR rats showed increased *Dnmt1* mRNA expression only in the CA1 region and at PD14 only. These results indicate complex age and region specific alterations of *Dnmt1* mRNA within the hippocampus that may underlie susceptibility to develop an anxious phenotype.

Further work using selectively bred strains found that DNA methylation of the *fibroblast growth factor-2* (*Fgf2*) gene and H3K9me3 binding at this gene are elevated in the hippocampus of anxiety-prone animals, while the opposite profile is found in less anxious animals (Chaudhury *et al.*, 2014). FGF2 plays a crucial role in hippocampal neurogenesis and may have anxiolytic properties. As noted above, H3K9me3 is a repressor of gene activity. As such, the finding that the association of H3K9me3 with *Fgf2* is higher in anxious animals is consistent with the low expression of *Fgf2* in these animals. The association of H3K9me3 with the *Nr3c1* gene is lower, consistent with higher levels of *Nr3c1* expression in bLR rats. Further, neonatal FGF2 administration decreases total hippocampal H3K9me3 levels in bLR rats compared to bLR vehicle-treated rats. Hippocampal *Fgf2* knockdown eliminates anxiety differences between the strains and increases levels of H3K9me3 in bHR's. In these animals, the protective mechanisms of *Fgf2* are attenuated by high association of H3K9me3 to *Fgf2* and low association to *Nr3c1* at baseline. These basal differences may contribute to the development of an anxious phenotype.

Early-life nutrition affects anxiety-like behaviors in animals. Three-weeks of a methyl-donor-deficient diet exposure, beginning at three weeks of age, reduced anxiety-like behavior on the EPM and decreased the gene expression of *Dnmt3a* and *Dnmt3b*, but not *Dnmt1*, in the hippocampus (Ishii *et al.*, 2014). After this exposure, animals transitioned to a diet supplemented with methyl donors and were assessed six weeks later. Although the methyl supplemented diet reversed alterations in gene expression, anxiety-like behaviors were increased in these animals. These results highlight the importance of the developmental

period at the time of perturbation. A poor nutritional diet in early life and into adulthood altered anxiety-like behaviors and both isoforms of *Dnmt3*. The switch to an improved diet rescued *Dnmt3a* expression, but heightened anxiety levels.

The hippocampus is particularly sensitive to elevated levels of corticosterone. A rodent model was used to mimic increased cortisol levels often seen in humans with anxiety disorders. Male adolescent mice exposed to corticosterone for four weeks displayed increased anxiety-like behavior as indicated by increased time spent in the closed arms of an EPM (Lee *et al.*, 2010, Yang *et al.*, 2012). This was accompanied by decreased DNA methylation of the *FK506 binding protein 5 (Fkbp5)* gene in whole hippocampal tissue and in dentate gyrus, a sub-region of hippocampus. Additionally, corticosterone treated mice had decreased *Dnmt1* mRNA expression levels in hippocampal tissue. FKBP5 modulates glucocorticoid activity by decreasing glucocorticoid receptor affinity for glucocorticoids. Chronic corticosterone exposure may mediate decreased DNA methylation of *Fkbp5* via decreased expression of *Dnmt1*. And, decreased DNA methylation of *Fkbp5* may facilitate glucocorticoid resistance in mice contributing to expression of anxiety-like behaviors.

Chronic unpredictable stress induces anxiety and alters enzymes involved in histone acetylation. Specifically, CUS decreases ambulatory and rearing behavior in the open field in adult male rats (Liu *et al.*, 2014). Anxiety-like behaviors are accompanied by decreased acetylation of H3K9 and histone H4 at lysine 12 (H4K12) and increased HDAC5 expression in the hippocampus. Interestingly, anxiety-like behaviors are attenuated when animals were administered an HDAC5 inhibitor. The HDAC5 inhibitor also prevents CUS-induced decreases in H3K9 and H4K12 acetylation. Further studies should examine the association between decreased histone acetylation and gene transcription to understand how this relationship contributes to an anxious phenotype. The efficacy of HDAC inhibitors in clinical populations for the treatment of anxiety disorders remains to be elucidated.

6.2. Amygdala

Preclinical research examining the role of miRNAs in anxiety disorders has been limited. Mice subjected to restraint stress for 30-min displayed up to a three-fold decrease or increase in several miRNAs (Haramati *et al.*, 2011). Bioinformatic analysis determined that the majority of stress-related genes altered after restraint stress were targeted by the miR-34 family. Gene targets included *corticotropin releasing hormone receptor 1 (Crhr1)*, *metabotropic glutamate receptor 7*, *5-hydroxytryptamine receptor 2C*, *GABA_A receptor $\alpha 4$* , and *Bdnf*. Within the miRNA-34 family, miR-34c had the highest expression after acute restraint stress and was upregulated two weeks after chronic social defeat stress. Artificial overexpression of miR-34c prevented the development of anxiety-like behaviors 24 h after acute restraint stress. Mice with overexpressed miR-34c increased their entries into and had a shorter latency to enter the light chamber. These mice traveled a larger percentage of their distance in and spent more time in the open arms on the EPM relative to controls. Thus, the miRNA-34 family bi-directionally regulates the activity of several genes involved in the stress response that may facilitate anxiety-like behaviors after exposure to acute or chronic stressors.

Chronic stress in selectively bred strains alters behavior and DNA methylation of the *Crhr1* gene. At baseline, low-anxiety mice had low *Crhr1* mRNA expression while high-anxiety mice displayed high *Crhr1* expression. *Crhr1* DNA methylation did not differ between strains at baseline. Low-anxiety mice exposed to mild stressors from PD 15–42 displayed decreased time spent in the light compartment of the light-dark box and in the open arms of the EPM (Sotnikov *et al.*, 2014). These animals also show increased *Crhr1* expression and increased *Crhr1* DNA methylation at the CpG 1 site in the basolateral amygdala. High-anxiety mice exposed to environmental enrichment displayed decreased *Crhr1* expression, increased DNA methylation of *Crhr1*, but showed decreased anxiety-like behavior. These results provide further evidence that genetic predispositions to anxiety-like behaviors can be shifted by environmental manipulations via *Crhr1* mechanisms.

Epigenetic differences within the amygdala and between selectively bred strains may be time-dependent and sub region-specific. For example, high-anxiety bred rats have decreased *Dnmt1* mRNA expression in the basolateral and lateral amygdala, with a trend seen in central nucleus, while there was increased expression in the medial nucleus only at PD7 (Simmons *et al.*, 2013). Additionally, levels of the repressive H3K9me3 were higher in the amygdala of high-anxiety bred animals (Chaudhury *et al.*, 2014). There was a higher association of H3K9me3 with *Fgf2* in anxious animals, consistent with the low expression of *Fgf2* in the amygdala. Anxious animals had a lower association of H3K9me3 with the *Nr3c1* gene, consistent with higher levels of *Nr3c1* expression in the anxious animals. FGF2 administration early in life did not alter H3K9me3 levels in the amygdala of anxious animals, but increased H3K9me3 levels in less anxious animals relative to their vehicle-treated controls. Highly anxious animals may be displaying their distinct phenotype due to altered regulation of pro anxiety-related genes via decreased *Dnmt1* mRNA expression, in combination with repression of anxiety-resilient genes via increased H3K9me3.

6.3. Nucleus Accumbens

Basal differences between low- and high-anxiety bred rats also are found in the NAc (Chaudhury *et al.*, 2014). Total levels H3K9me3 are higher in the NAc of high-anxiety bred animals. Like in the hippocampus and amygdala, the association of H3K9me3 with *Fgf2* is higher in the high-anxiety animals. In contrast, the association of H3K9me3 with the *Nr3c1* gene is lower and this association may contribute to the higher levels of *Nr3c1* expression in the high-anxiety animals. *Fgf2* administration early in life does not alter H3K9me3 levels in the NAc of anxious animals, but *Fgf2* treatment increases total H3K9me3 levels in low-anxiety animals. Higher *Nr3c1* and lower *Fgf2* expression may play a role in developing an anxiety-like phenotype via altered H3K9me3 mechanisms. Because elevated levels of H3K9me3 are found in three brain regions (hippocampus, amygdala, and NAc) of high-anxiety animals, this histone modification may have more widespread repressive effects.

6.4. Hypothalamus

Anxiety-inducing social defeat stress alters epigenetic regulation of *Crf* in the hypothalamus. Two weeks after their last social defeat session, defeated mice displayed increased *Crf* mRNA expression and decreased DNA methylation of the promoter region in the PVN of the hypothalamus (Elliott *et al.*, 2010). One hour after the last defeat session, mice showed

decreased *Dnmt3b* and *Hdac2* expression, as well as increased *Growth Arrest and DNA-Damage-Inducible, Beta* (*Gadd45b*) gene expression. GADD45B may be necessary for demethylation at the promoter region of genes, including *Crf*. Interestingly, decreases in *Hdac2* gene expression persisted for up to two weeks after the last defeat session. A three-week treatment with the antidepressant imipramine after social defeat prevented stress-induced elevations in *Crf* mRNA and DNA hypomethylation of the *Crf* promoter in the hypothalamus. In a similar vein, chronic corticosterone treatment increased anxiety-like behavior and decreased DNA methylation of *Fkbp5*, in the hypothalamus of male mice (Lee *et al.*, 2010). Taken together, these results suggest corticosterone-inducing stimuli may trigger epigenetic mechanisms that facilitate both decreased DNA methylation of corticosterone precursor factors (*Crf*) and decreased methylation of corticosterone regulatory processes (*Fkbp5*).

6.5. Raphe Nuclei

The majority of cell bodies in the raphe nuclei are serotonergic and altering this neurotransmitter system is associated with mood and anxiety disorders. Issler and colleagues (2014) examined the role of miRNAs in modulating serotonin activity. miR-135a repressed translation of both the *serotonin receptor 1A* (*Htr1a*) and the *5-HTT* genes. Treatment with a selective serotonin reuptake inhibitor (SSRI) increased miR-135 in the raphe nuclei of mice. Interestingly, social defeat stress did not alter miR-135 levels within this region. Animals with overexpressed miR-135 in serotonin neurons of this region displayed no baseline anxiety-like behaviors, but did show resiliency to social defeat-induced anxiety. These mice spent more time, made more visits, and traveled longer distances in the light compartment of the light/dark test compared to controls. They also displayed less anxiety-like behavior on the EPM after social defeat. Conversely, miR-135 knockdown animals showed increased anxiety-like behavior on the EPM and light/dark tests. Thus, miR-135 in serotonergic raphe neurons may provide a protective mechanism against social defeat stress-induced anxiety and may be a possible secondary mechanism of action of SSRI's.

6.6. Prefrontal Cortex

In the adult PFC, histone-lysine N-methyltransferase 2A (KMT2A) encoded by the *Kmt1a* (*Mll1*) gene is a subunit of the MLL1/MLL complex that regulates the levels of H3K4me3 at promoter regions of a small subset of genes implicated in cognition and emotion, as well as the acetylation of lysine 16 of histone H4 (H4K16ac). Tri-methylation of H3K4 at this site is associated with genes transcriptionally activated. Mice bred with PFC-specific knockdown of *Mll1* displayed elevated anxiety-like behaviors indicated by increased aversion to the bright compartment in the light/dark box test and reduced time spent in the center of the open field (Jakovcevski *et al.*, 2015). A separate group of mice displayed similar anxiety levels when the *Kmt1a* (*Mll1*) gene was deleted from PFC neurons in adulthood. Additionally, *Kmt1a* (*Mll1*) -deficient mice displayed lower binding of H3K4me3 at specific promoter sequences accompanied by decreased levels of corresponding RNA's. Several of these genes are altered by deficits in H3K4me3 and mediate cortical development and neuron differentiation. Given this information, KMT2A deficiency in the PFC may induce susceptibility to anxiety-behavior by decreasing H3K4me3 transcriptional activation. Relatedly, knockdown of the long ncRNA *Gomafu*, that may be involved in alternative

splicing (Barry *et al.*, 2014) in the prelimbic region of the PFC decreased time spent and distance traveled in the center of the open field (Spadaro *et al.*, 2015). Further *in vitro* and *in vivo* work provided support that *Gomafu* is a negative regulator of a crystalline beta family member CRYBB1, a protein that mediates cellular homeostasis in response to stressors. Dysregulation of CRYBB1 has been associated with schizophrenia and autism. Rodents with *Crybb1* knockdown in the PFC showed decreased anxiety-like behavior. *Gomafu* expression may be a protective mechanism preventing elevated levels of *Crybb1*. Overall, these findings suggest a significant role of PFC-associated epigenetic changes in anxiety-like behavior.

7. Human Studies

Compared to animal studies, there are fewer studies of anxiety in humans. Yet, examining the epigenetics of anxiety disorders may lead to a better understanding of mechanisms and thereby help direct intervention or prevention strategies (Table 6). DNA methylation studies have been conducted in adults with anxiety symptoms (Murphy *et al.*, 2015), social anxiety disorder (Ziegler *et al.*, 2015), and panic disorder (Domschke *et al.*, 2012, Domschke *et al.*, 2013). In anxious and non-anxious adults, as determined by scores on the Hospital Anxiety and Depression Scale-Anxiety (HADS-A), global DNA methylation levels were higher in anxious adults relative to non-anxious adults (Murphy *et al.*, 2015). Further, the expression of the DNA methyltransferases DNMT1/3A were positively correlated with HADS-A scores in the anxious group, with increased expression related to higher anxiety scores. In patients with specific anxiety disorders, particularly panic and social anxiety disorders, DNA hypomethylation was identified.

Adults with panic disorder showed *glutamate decarboxylase 1 (GAD1)* DNA hypomethylation primarily at three CpG sites in the promoter, as well as at one site in intron 2 compared to healthy controls (Domschke *et al.*, 2013). *Monoamine oxidase A (MAOA)* hypomethylation also was found in females with panic disorder but not in males (Domschke *et al.*, 2013). *Norepinephrine transporter (SLC6A2, NET)* DNA hypermethylation was seen in adults with panic disorder (Esler *et al.*, 2006, Esler *et al.*, 2008). Adults with social anxiety disorder, exhibited *oxytocin receptor (OXTR)* DNA hypomethylation suggesting an *OXTR* compensatory mechanism in this disorder (Ziegler *et al.*, 2015). These studies demonstrate that DNA methylation differences exist in specific anxiety disorders and such differences may serve as biomarkers and as well as sites of treatment intervention.

Methylation changes were found in children with an anxiety disorder after a manualized cognitive-behavioral therapy (CBT) intervention (Roberts *et al.*, 2014). The *5-HTT* DNA methylation change differed between treatment responders versus non-responders over the course of the intervention. This effect was primarily driven by a single CpG site in the *5-HTT* gene in which responders had an increase in DNA methylation and non-responders had a decrease in DNA methylation. This demonstrates methylation changes in response to psychotherapy only and that biological changes co-occur with improvements in symptomatology following a behavioral intervention.

Pharmacological intervention may counteract some epigenetic processes or cause epigenetic changes themselves. The preferred first-line of treatment of anxiety disorders are selective

serotonin reuptake inhibitors (SSRIs) (Nutt, 2005) that have direct and indirect effects on the genes implicated above (e.g., *5-HTT*, *NET*). SSRIs block the reuptake of serotonin, thus increasing the availability of serotonin in the synapse. Further, SSRIs can induce miR-135a (Issler *et al.*, 2014) (see Section 6.5). Perhaps, since *5-HTT* methylation is increased by successful CBT (Roberts *et al.*, 2014), SSRIs also may increase *5-HTT* methylation. Thus, an epigenetic intervention that increases *5-HTT* promoter methylation may alleviate anxiety by increasing serotonin transporter levels that subsequently would decrease serotonin levels in the synapse. In addition to SSRIs, monoamine oxidase inhibitors (MAOIs), such as phenelzine and tranylcypromine increase synaptic availability of serotonin, as well as norepinephrine (also called noradrenaline) and dopamine (Nutt and Ballenger, 2003). Similar to SSRIs, MAOIs could counteract the *MAOA* hypomethylation found in females with panic disorder (Domschke *et al.*, 2013). Further research is needed to understand the impact of pharmacological treatments on DNA methylation in those with anxiety disorders.

8. Epigenetic and Genetic Interactions

Anxiety disorders show strong familial associations that reflects, in part, genetic (DNA) contributions. However, the transmission of anxiety-like phenotypes within families may also occur if the offspring are under stressful family experiences consistent with the large preclinical literature in which stress exposures, particularly those experienced early in life, have long-term consequences on anxiety-like behaviors and on epigenetic processes (see Section 5). Increasingly, epigenetic changes are speculated to be passed on to subsequent generations via a heritable process that occurs during mitosis and meiosis (Reik, 2007). Although much epigenetic information is erased during post-fertilization processes, transmission of epigenetic alterations that were environmentally induced in the parental generation are observed in the offspring and even in the grand offspring (see Section 4). Early life environmental insults may affect the epigenome of females that, in turn, can affect the maternal care provided to their offspring and lead to epigenetic changes. Additionally, maternal care can also be influenced by the phenotypical state of the father during the mating encounter. For instance, a dam may change her behavior toward offspring due to a sexual encounter with a stressed male, thereby contributing to a change in the epigenetic profile of her offspring. Indeed, this type of non-genomic transmission of phenotypes has been demonstrated in rodents (Champagne *et al.*, 2003). However, males can transmit effects to their offspring that persist into subsequent generations. Such effects occur through the germline. For example, male mice with maternal separation experience show deficits in social behavior, an anxiety-like phenotype, and altered serotonergic function (Franklin *et al.*, 2011). The sperm of these males have increased DNA methylation in *MeCP2* and decreased DNA methylation of *Crf2* (Franklin *et al.*, 2010) and these epigenetic changes are also found in the sperm and cortex of their offspring (Franklin *et al.*, 2011). Thus, anxiety disorders may show familial associations due to inherited epigenetic effects and/or to maternal behaviors towards the offspring.

The role of epigenetics in anxiety disorders should be viewed within the context of an individual's genetic background. Indeed, the preclinical literature reveals strain-dependent differences in the development of anxiety-like behaviors or in epigenetic changes after exposure to the same stressful stimuli. This suggests that genetic contributions may mediate

susceptibility or resiliency to develop anxiety disorders after stress. In humans, the *5-HTT* gene has a variable number tandem repeat (*5-HTTLPR*) in its promoter region that alters serotonin levels and contributes to the individual's sensitivity to stress (Lesch *et al.*, 1996, Murphy *et al.*, 2004). Depressive symptoms are more highly correlated with the number of stressful life events experienced in those with a greater number of the short *5-HTTLPR* alleles (Caspi *et al.*, 2003). Short allele carriers also show attenuations in their perceived limitations to cope with effects of stress (Fox *et al.*, 2011, Graham *et al.*, 2013). Response to CBT in adults with post-traumatic stress disorder is more positive in long/long *5-HTTLPR* genotype patients (Bryant *et al.*, 2010). This genetic difference may interact with epigenetic processes. Depressed mood associates with *5-HTT* promoter hypomethylation (Devlin *et al.*, 2010). As discussed in Section 7, children who respond to CBT increased their *5-HTT* promoter methylation levels, while those that did not respond had decreased methylation (Roberts *et al.*, 2014). Moreover, individuals with *5-HTT* promoter hypomethylation and the long/long *5-HTTLPR* genotype show lower cortisol levels in response to an acute stressor, while those carrying one or two hypomethylated short alleles have an enhanced response (Alexander *et al.*, 2014). In contrast, an intermediate response is seen in those with hypermethylation the *5-HTT* promoter, regardless of genotype. Thus, we speculate that those with the long/long *5-HTTLPR* genotype and hypermethylation of these alleles will have higher levels of 5-HTT and low responsiveness to their environment, which, in turn, will produce lower levels of anxiety symptoms or behaviors. On the other hand, short allele carriers with hypermethylation will produce low levels of 5-HTT, be more responsive to their environment, and therefore, may become anxious when faced with prior stressful experiences. This example of interactions between environmental stimuli and its consequential epigenetic effects with genotype is illustrated in Fig. 1 and demonstrates how both behavioral and pharmacological treatment strategies may need to be tailored to the individual based on their genotype, methylation status, and prior stressful experiences.

9. Future Directions

Research on the contributions of epigenetic processes in the susceptibility or resiliency to develop behavioral disorders, including anxiety, is in its infancy. As such, the literature is limited and there are many gaps of knowledge. Overall, the majority of epigenetic modifications identified to date show that anxiety-like phenotypes involve genes that regulate the HPA axis, neurotransmitter systems, and neuroplasticity. Some studies revealed sex differences; however, a more systematic approach involving both sexes is imperative to further understand potential sex or gender effects given that a disproportionate number of women suffer from anxiety disorders (McLean *et al.*, 2011). Additionally, the time of perturbation can differentially disturb the epigenome. Identifying the mechanisms underlying these age-dependent differences will provide a more complete understanding of the etiology of anxiety disorders and, perhaps suggest valuable prevention strategies. Thus far, the foci of research conducted at the preclinical (animal) and clinical (human) levels are not well-aligned. Relatively few preclinical studies explored the epigenetic effects of currently used pharmacological treatments for anxiety disorders. In the sparse human literature, several studies focused on patients with panic disorders with very little focus on other subsets of anxiety. Human research that closely examines each specific type of anxiety

has the potential to elucidate unique epigenetic changes that may result in more efficacious prevention and treatment strategies for specific anxiety disorders.

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Highlights

- Anxiety disorders may associate with epigenetic alterations
- Environmental insults affect the epigenome and anxiety-like behaviors in animals
- Many changes are specific to brain region and to time of perturbation
- Research in humans is sparse and needs to align better with preclinical studies
- Information about epigenetic processes may inform prevention and treatment strategies

Figure 1.

Schematic representation of the role of early-life stress and *5-HTTLPR* genotype has on response to stress in the development of anxiety. A: Early-life stress (fuchsia lightning bolt) could produce DNA hypermethylation (closed circles) of the *5-HTT* (*Slc6a2*) gene promoter. In an individual with the S'S' *5-HTTLPR* genotype (open box) this would cause low *5-HTT* gene expression resulting in low pre-synaptic 5-HTT levels and high basal 5-HT levels (orange ovals). These individuals would be hyper-responsive to stress (red lightning bolt) and have a high level of anxiety (!!!). B: Without early-life stress, individuals with the S'S' *5-HTTLPR* genotype (open box) could have DNA hypomethylation (open circles) of the *5-HTT* gene promoter, intermediate levels of *5-HTT* gene expression and 5-HT (orange ovals). These individuals would have a moderate response to stress (red lightning bolt) and a moderate level of anxiety (!!). C: Early-life stress (fuchsia lightning bolt) could cause DNA methylation (closed circles) of the *5-HTT* gene promoter. Individuals with the L'L' *5-HTTLPR* genotype (stripped box) and DNA hypermethylation would have moderate *5-HTT* gene expression resulting in intermediate 5-HTT and basal 5-HT levels. These individuals would have an intermediate response to stress (red lightning bolt) and a moderate level of anxiety (!!). D: Individuals without early-life stress could have hypomethylation of the *5-HTT* gene promoter (open circles). A combination of the L'L' *5-HTTLPR* genotype (stripped box) and DNA hypomethylation would lead to high *5-HTT* gene expression resulting in high 5-HTT and low basal 5-HT levels. These individuals would be hypo-responsive to stress (red lightning bolt) and show a low level of anxiety (!).

Table 1

Genes studied in the epigenetics of anxiety

Gene	Product	Species	Function
<i>Avp</i>	Arginine Vasopressin	Rodent	Posterior pituitary hormone with role in cognition, maternal behavior, etc.
<i>Bcl11a</i>	B-Cell CLL/Lymphoma 11A	Rodent	Zinc finger protein involved in neuroplasticity
<i>Bdnf</i>	Brain-derived neurotrophic factor	Rodent	Growth factor involved in neuroplasticity
<i>Crh</i>	Corticotrophin releasing hormone	Rodent	Stress response hormone
<i>Crhr1</i>	Corticotropin releasing hormone receptor 1	Rodent	G-protein coupled receptor (GPCR) for corticotrophin releasing hormone
<i>Crybb1</i>	Crystallin, beta B1	Rodent	Mediates cellular homeostasis in response to stressors
<i>Egr1</i>	Early growth response 1	Rodent	Transcriptional regulator with role in neuroplasticity
<i>Fgf2</i>	Fibroblast growth factor 2	Rodent	Growth factor involved in neuroplasticity
<i>Fkbp5</i>	FK506 binding protein 5	Rodent	Interacts with corticoid receptor complexes
<i>Gad1</i>	Glutamate decarboxylase 1	Rodent	Synthesis of γ -aminobutyric acid from L-glutamic acid
<i>GAD1</i>		Human	
<i>Gadd45b</i>	Growth arrest and DNA-damage-inducible, beta	Rodent	Stress response by activation of the p38/JNK pathway
<i>Htr1a</i>	5-HT _{1A} receptor	Rodent	Inhibitory GPCR mediating serotonin neurotransmission
<i>Jag1</i>	Jagged1	Rodent	Cell surface receptor involved in Notch signaling
<i>Kap1 (Trim28)</i>	KRAB-associated protein 1	Rodent	Transcriptional corepressor (Tripartite motif containing 28)
<i>MAOA</i>	Monoamine oxidase A	Human	Catalyzes oxidative deamination of amines (e.g., serotonin, dopamine, and norepinephrine)
<i>Kmt2a</i>	Lysine (K)-specific methyltransferase 2A	Rodent	Regulates H3K4me3 activity
<i>Nr3c1</i>	Nuclear receptor subfamily 3, group C, member 1	Rodent	Glucocorticoid receptor and transcriptional activator (glucocorticoid receptor)
<i>Nr4a1</i>	Nuclear receptor subfamily 4, group A, member 1	Rodent	Transcription factor involved in neuroplasticity (nerve growth factor IB)
<i>Ntsr1</i>	Neurotensin receptor 1	Rodent	GPCR with role in hypertension, thermal regulation, antinociception, etc.
<i>Oprm1</i>	μ -opioid receptor	Rodent	GPCR receptor for opioids and is involved in analgesia, reward, emotions, stress, etc.
<i>OXTR</i>	Oxytocin receptor	Human	GPCR with role in attachment and lactation
<i>Peg3</i>	Paternally-expressed 3	Rodent	Imprinted transcription factor gene involved in fetal growth and nurturing behaviors

Gene	Product	Species	Function
<i>Pomc</i>	Proopiomelanocortin	Rodent	Precursor of peptides involved in steroidogenesis, stress, energy homeostasis, immune modulation, etc.
<i>Rln</i>	Reelin	Rodent	Glycoprotein with role in synaptic plasticity and neuronal migration
<i>SLC6A4</i>	Solute carrier family 6, member 4	Human	Transports serotonin from synaptic cleft into
<i>Slc6a4</i>	(serotonin transporter, 5-HTT)	Rodent	presynaptic neuron
<i>SLC6A2</i>	Solute carrier family 6, member 2	Human	Transports norepinephrine from synaptic cleft into (norepinephrine transporter, NET) presynaptic neuron
<i>Syn1</i>	Synapsin I	Rodent	Neuronal phosphoprotein on cytoplasmic surface of synaptic vesicles that may modulate neurotransmitter release
<i>Mkri3</i>	Makorin ring finger protein 3	Rodent	Paternally imprinted gene encoding a putative ribonucleoprotein
<i>Pcdhb6</i>	Protocadherin beta 6	Rodent	Integral plasma membrane proteins with role in determining neural connections

Table 2

Preclinical models of anxiety

Test	Indices of Anxiety-like Behavior	Reference
Elevated Plus Maze (EPM)	↑ time/entries into closed arms ↓ time/entries into open arms	(Montgomery, 1955)
Open Field	↓ time/entries into center zone ↑ time/entries in peripheral zones	(Hall and Ballachey, 1932)
Light-Dark Box	↑ time/entries into dark chamber ↓ time/entries into light chamber	(Crawley and Goodwin, 1980)
Novelty-induced Hypophagia	↑ latency to feed	(Dulawa and Hen, 2005)
Novel Environment (stickleback fish)	↑ thigmotaxis	(Maximino <i>et al.</i> , 2010)

Preconception and prenatal manipulations associated with anxiety behaviors and epigenetic modifications

Table 3

Brain region	Manipulation	Primary findings	Findings by sex		Species	Reference
			Males	Females		
Hypothalamus	Prenatal restraint stress	↓ <i>Crh</i> methylation	✓	✓	SD rats	(Xu et al., 2014)
Cortex	Low omega-3 diet & western diet	↑ <i>Bdnf</i> methylation	✓	--	SD rats	(Tyagi et al., 2015)
	Prenatal restraint stress	↑ <i>Reelin</i> methylation	✓	✓	SD rats	(Palacios-Garcia et al., 2015)
	Paternal cocaine exposure	↑ association of H3 acetylation w/ <i>Bdnf</i>	✓	ns	SD rats	(Vassoler et al., 2013)
	Paternal ethanol exposure	↑ <i>Peg3</i> methylation	ns	✓	KM mice	(Liang et al., 2014)

✓ = Significant; -- = Not Included; ns = Not Significant; SD = Sprague Dawley; KM = Kunming.

Table 4

Early-life manipulations associated with anxiety behaviors and epigenetic modifications

Brain region	Manipulation	Primary findings	Findings by sex		Species	Reference
			Males	Females		
Hippocampus	Maternal separation	↑ <i>Avp</i> methylation	✓	ns	C57BL/6J &	(Kember et al., 2012)
		↓ <i>Nr4a1</i> methylation	✓	✓	DBA/2J mice	
		↑ <i>Nr3c1</i> methylation	✓	✓	C57BL/6J mice	
	Low maternal care	↑ <i>Nr3c1</i> methylation	✓	✓	DBA/2J mice	(Weaver et al., 2004)
		↓ H3K4 acetylation	✓	✓	LE rats	
Amygdala		↓ <i>Nr3c1</i> methylation	--	✓	LE rats	(Pan et al., 2014)
	Litter gender composition	↑ <i>Nr3c1</i> methylation	ns	✓		(Kosten et al., 2014)
		↑ <i>Bdnf</i> methylation	✓(ss)	ns	SD rats	
	Maternal separation	↑ <i>Oprm1</i> methylation	✓(ss)	✓(ss)	SD rats	(Hao et al., 2011)
		↑ <i>Ntsr1</i> methylation	✓	--	SD rats	(Toda et al., 2014)
Striatum		↑ <i>Syn1</i> methylation	✓	✓	SD rats	(Park et al., 2014)
	Low maternal care	↑ <i>Dnmt1</i> expression	✓	✓	SD rats	(Zhong et al., 2015)
		↑ Total methylation	✓	✓		
	Anxious temperament	↑ <i>BCL11A</i> & <i>JAG1</i> methylation	✓	--	RM monkeys	(Alisch et al., 2014)
	Litter gender composition	↑ <i>Nr3c1</i> methylation	ns	✓(ss)	SD rats	(Kosten et al., 2014)
Hypothalamus		↓ <i>Egr1</i> methylation	✓(ss)	ns		
	Maternal separation	↑ <i>Oprm1</i> methylation	✓(ss)	ns	SD rats	(Hao et al., 2011)
		↓ <i>Avp</i> methylation	✓	--	C57BL/6N mice	(Murgatroyd et al., 2009)
Pituitary	Maternal separation	↓ <i>Crh</i> methylation	✓	✓	SD rats	(Chen et al., 2012)
Prefrontal cortex	Maternal separation	↓ <i>Pomc</i> methylation	✓	--	C57BL/6N mice	(Wu et al., 2014)
	Low maternal care	↑ <i>Bdnf</i> methylation	✓	✓	LE rats	(Roth et al., 2009)
Whole brain	Low Paternal Care	↓ <i>Dnmt3a</i> expression	✓	✓	SB fish	(McGhee and Bell, 2014)
Blood	Variable Foraging Demand	↑ <i>5-HTT</i> methylation	--	✓	RM monkeys	(Kinnally et al., 2011)
		↑ global methylation				

✓ = Significant; NS = Not Significant; -- = Not Included; SS = Single Sex; SD = Sprague Dawley; TG = Transgenic; LE = Long Evans; RM = Rhesus Macaques; SB = Stickleback.

Table 5

Adult and juvenile manipulations associated with anxiety behaviors and epigenetic modifications

Brain region	Manipulation	Primary findings	Findings by sex		Species	Reference
			Males	Females		
Hippocampus	<i>Kap1</i> knockout	↓ H3K9me3 at <i>Mktn3</i> & <i>Pcdh96</i>	✓	--	TG mice	(Jakobsson et al., 2008)
		↑ H3 & H4 acetylation at <i>Mktn3</i> & <i>Pcdh96</i>	✓	--		
	High anxiety strain	↓ <i>Dnmt1</i> expression in DG & CA3	✓	--	bLR-HR rats	(Simmons et al., 2013)
		↑ <i>Dnmt1</i> expression in CA1	✓	--		
	High anxiety strain	↑ <i>Fgf2</i> methylation	✓	✓	bLR-HR rats	(Chaudhury et al., 2014)
		↑ H3K9me3	✓	✓		
	Methyl-donor-deficient diet	↓ <i>Dnmt3a</i> & <i>Dnmt3b</i> expression	✓	--	C57BL/6J mice	(Ishii et al., 2014)
	Corticosterone administration	↓ <i>Fkbp5</i> methylation	✓	--	C57BL/6J mice	(Yang et al., 2012)
		↓ <i>Dnmt1</i> expression	✓	--		
	Chronic unpredictable stress	↓ H3K9 & H4K12 acetylation	✓	--	SD rats	(Liu et al., 2014)
		↑ <i>Hdac5</i> expression	✓	--		
Amygdala	miR-34c overexpression	↑ anxiolytic behaviors	✓	--	TG & C57BL/6J mice	(Haramati et al., 2011)
	Chronic mild stress	↑ <i>Chtr1</i> methylation	✓	--	Inbred strain (CD1 background)	(Somikov et al., 2014)
	High anxiety strain	↓ <i>Dnmt1</i> expression in BL & L	✓	--	bLR-HR rats	(Simmons et al., 2013)
		↑ <i>Dnmt1</i> expression in MN	✓	--		
	High anxiety strain	↑ <i>Fgf2</i> methylation	✓	✓	bLR-HR rats	(Chaudhury et al., 2014)
		↑ H3K9me3	✓	✓		
Nucleus Accumbens	High anxiety strain	↑ H3K9me3	✓	✓	bLR-HR rats	(Chaudhury et al., 2014)
Hypothalamus	Social defeat stress	↓ <i>Crf</i> methylation	✓	--	C57BL/6J mice	(Elliott et al., 2010)

Brain region	Manipulation	Primary findings	Findings by sex		Species	Reference
			Males	Females		
			✓	--		
		↓ <i>Dnmt3b</i> & <i>Hdac2</i> expression				
	Corticosterone administration	↓ <i>Fkbp5</i> methylation	✓	--	C57BL/6J mice	(Lee et al., 2010)
Raphe Nuclei	miR-135 knockdown	↑ anxiogenic behaviors	✓	--	C57BL/6 & TG mice	(Issler et al., 2014)
Prefrontal Cortex	<i>Mlll</i> deletion	↓ H3K4me3 at multiple promoter regions	✓	✓	TG mice (C57BL/6J background)	(Iakovcevski et al., 2015)
	<i>Gomafu</i> knockdown	↑ anxiolytic behaviors	✓	--	C57BL/6 mice	(Spadaro et al., 2015)

✓ = Significant; -- = Not Included; SD = Sprague Dawley; TG = Transgenic; bLR-HR = Bred Low Responders and Bred High Responders.

Table 6

Epigenetic modifications associated with anxiety disorders in humans

Sample collection	Age	Anxiety diagnosis/symptoms	Primary findings	Findings by sex		Reference
				Males	Females	
Blood	Adult	Hospital Anxiety Depression Scale - Anxiety score > 8	↑ Global DNA methylation	✓	✓	(Murphy et al., 2015)
Blood	Adult	Panic disorder	↓ <i>GAD1</i> methylation	✓	✓	(Domschke et al., 2013)
Blood	Adult	Panic disorder	↓ <i>MAOA</i> methylation	✓	✓	(Domschke et al., 2012)
Blood	Adult	Social anxiety disorder	↓ <i>OXTR</i> methylation	✓	✓	(Ziegler et al., 2015)
Blood	Adult	Panic disorder	↑ <i>NET</i> methylation	✓	✓	(Esler et al., 2006; Esler et al., 2008)
Buccal	Children (6–13 years of age)	Any anxiety diagnosis	↓ <i>SERT</i> methylation in nonresponders to CBT	✓	✓	(Roberts et al., 2014)
			↑ <i>SERT</i> methylation in responders to CBT	✓	✓	

CBT = Cognitive Behavior Therapy; ✓ = Significant.