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The Epigenetics of Anxiety Pathophysiology: A DNA Methylation and Histone Modification Focused Review

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Abstract

Anxiety is one of the most common psychiatric disorders diagnosed in the USA today. Like all mental illnesses, anxiety pathology includes genetic, molecular, somatic, and behavioral characteristics. Specific brain regions implicated in anxiety include the prefrontal cortex, amygdala, hippocampus, and hypothalamus. Together, these regions regulate fear-related learning and memory processes, and are innervated by neuronal projections that utilize glutamate and gamma-aminobutyric acid (GABA) as neurotransmitters. Neurotrophic factors such as brain derived neurotrophic factor (BDNF), are also implicated in anxiety. This review discusses the neuroepigenetics of the anxiety phenotype. While studying such changes is limited to post-mortem brain studies or peripheral tissue acquisition in humans, the use of animals to model anxiety phenotypes has made epigenetic research possible. In this review, we summarize and discuss a plethora of DNA methylation, histone modification, and associated gene expression differences underscoring the anxiety phenotype.

Findings we outline include expression changes of various DMNTs and changes in histone modifications that affect the hypothalamic pituitary adrenal axis and stress response as well as GABA, glutamate, and BDNF signaling in the PFC, amygdala, hypothalamus, and hippocampus. Furthermore, there have been studies showing that anxiety behaviors and biological scars from stress can be reversed using HDAC inhibitors, and we discuss ideas for the future of treatment.

In this review, we hope that by compiling much of the data pertaining to DNA methylation and histone modifications in vivo animal studies, that we are able to highlight potential avenues for future research despite existing limitations.

Introduction

Coined in 1942 by Conrad Waddington, epigenetics, from the Greek 'epi' meaning over or above, genetics, is the accepted link between the environment's ability to influence an organism's genome in order to alter gene expression and thus, the observed phenotype (Tronick & Hunter, 2016). Epigenetics therefore accounts for the way environmental factors like stress, 'speak' to genes to modulate gene expression. In neuroepigenetics, these modifications alter brain plasticity, and may render an individual predisposed towards developing mental illnesses such as anxiety disorders (ADs) (Fig.1) (Schiele & Domschke, 2020). Epigenetic modifications do not change one's DNA sequence, rather, they modulate levels of gene expression via four main mechanisms: DNA methylation, histone modifications, noncoding RNA interactions and nucleosome positioning (Schiele & Domschke, 2017). In this review, the more prominently studied of these mechanisms in anxiety research, DNA Methylation and Histone Modifications, are discussed in detail.

DNA Methylation

DNA methylation involves the covalent transfer of a methyl group from S-adenyl methionine (SAM) to the C-5 position of a cytosine ring of DNA to form 5-methylcytosine (5mC) (Moore et al., 2013). This reaction is catalyzed by a large class of enzymes known as DNA methyltransferases (DNMTs), composed of DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (Morris et al., 2016). Additionally, DNA methylation is mediated by a family of proteins that bind 5mC, including methyl-CpG-binding protein (MeCP2) (Martinowich et al.,

2003). Oftentimes, when methylation marks are present on gene promoters, usually in CpG islands, gene transcription is repressed (Jin et al., 2011).

Histone Modifications

In eukaryotic organisms, DNA is compacted into basic repeating units called nucleosomes which are composed of an octamer of the four core histone protein subunits: H2A, H2B, H3 and H4 (Grant, 2001). DNA is coiled around these histone proteins to form a nucleosome, and in turn, these nucleosomes are packed together to form chromatin. Each core histone carries a tail that extends into the space surrounding a nucleosome, providing sites for a wide variety of posttranslational modifications (Fig. 2). These modifications or markers include methylation, acetylation and phosphorylation, to name a few, and are catalyzed by specific enzymes (Grant, 2001). Histone markers interact with other proteins present in the nucleus to form complexes that shift chromatin's structure at specific sites along the genome, between heterochromatin (compactly packed, repressed transcription) and euchromatin (loosely packed, increased transcription) (Sun et al., 2013).

The acetylation state of histones is regulated by two classes of enzymes: histone acetyltransferases (HATs), which add acetyl groups to histone tails at lysine residues, and histone deacetylases (HDACs), which removes these acetyl groups. There are two types of HATs: type-A and type-B, with type-A being divided into three main families of HATs: Gcn5-related *N*-acetyltransferases (GNATs), MYST (named for a collection of genes) and CREB-binding protein (CBP/p300). (Bannister & Kouzarides, 2011). There are four classes of HDACs (Class 1-4), with HDACs of Class 1 and 2 assuming more major roles in the nervous system (Abel & Zukin, 2008). Class 1 consists of HDAC1 and HDAC2, while Class 2 is composed of HDAC4, HDAC5 and HDAC7. Most often, acetylated histones are associated with

transcriptionally active chromatin, as it allows increased access of transcription factors to exposed gene promoters and transcription start sites (TSS), while deacetylated histones are associated with inactive transcription, though there are repressive acetyl marks (de Ruijter et al., 2003).

Histone methylation is regulated by histone methyltransferases (HMTs), which may add multiple methyl groups at lysine or arginine residues. One can therefore find histone tails that are either mono-, di- or trimethylated (Grant, 2001). Histone lysine methyltransferases (HKMTs) methylate histones at lysine residues, while protein arginine methyltransferases methylate histones at arginine residues (PRMTs) (Bannister & Kouzarides, 2011). Histone methylation can be either repressive or permissive, depending on the location and number of methyl groups as we will see in the studies discussed later.

AD Pathology

Anxiety disorder (AD) pathogenesis is multifactorial: development of an anxiety disorder involves biological, environmental and psychological factors. Early-life stressors (prenatal or postnatal), substance abuse in adolescence and adulthood, as well as genetics, influence one's risk for developing an AD, though it is understood that these factors do not entirely account for AD pathogenesis, and may also contribute to the development of other disorders such as mood and depressive pathologies (Schiele & Domschke, 2017). Thus, there has been an increased focus on identifying factors that contribute to an individual's resiliency or susceptibility towards developing an AD. In this review, we focus on summarizing differential DNA methylation and histone modification findings in anxiety models compared to control counterparts. Note that when studying anxiety in animal models, stress response is used as a proxy to study anxiety response.

When we consider the pathways involved in an anxiety or fear-conditioned response, the question arises: what factors influence memory formation, stimulus conditioning, and subsequent anxious behavior learned? The pathophysiology of AD demands that specific brain regions, their associated neurotransmitters, the Hypothalamic Pituitary Adrenal (HPA) Axis and its hormone components, all be considered when investigating anxiety from a genetic and epigenetic point of view (Bartlett et al., 2017). In humans, the brain is implicated in all psychiatric disorders. It is particularly responsive to stress and has the capacity for reversible structural changes (plasticity) that enables us to continuously adapt to the changing environment. Stress has been shown to induce such changes in various limbic system structures including the amygdala and the hippocampus. The amygdala is actively engaged in ambiguous situations and contributes to the shaping of perception and value representation, such as labeling an object or experience as 'rewarding' or 'aversive'. The hippocampus facilitates contextual fear learning, which underlies the anxiety-phenotype (Pessoa, 2010; Zhang et al., 2014). These neuronal changes include dendritic remodeling (expansion and contraction of dendritic trees), turnover of synaptic connections, and limited neuronal replacement via neurogenesis. Resilience associated with plasticity is often lost with age, resulting in impaired reversibility of these changes (Hunter & McEwen, 2013).

In the central nervous system (CNS), this plasticity is modulated by neurotrophic factors, which promote neuronal growth, survival and regeneration, and are commonly referred to as neurotrophins (Xiao & Le, 2016). One such example is brain derived neurotrophic factor, BDNF, which is a small protein encoded by the *BDNF* gene essential for neuronal growth, differentiation and the overall development of the CNS. This includes brain repair following injury and the formation of long-term memory, such as the consolidation of aversive memories

113 or constructive learning, all of which may contribute to the development of an AD (Mitte, 2008;
114 Cattaneo et al., 2016).

115 Additionally, brain regions implicated in fear and anxiety responses are interconnected
116 and capable of communicating with each other via the action of neurohormones and
117 neurotransmitters - all of which are potential targets for anxiety based epigenetic investigations.
118 Gamma-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the mature
119 mammalian brain and CNS. It is capable of binding to two main receptors: $GABA_A$ and $GABA_B$,
120 which are gated Cl^- channels (Valenzuela et al., 2011). On average, approximately a third of
121 CNS neurons utilize GABA as a neurotransmitter, particularly interneurons. GABAergic neurons
122 from the central amygdala (CeA), an integration center which converts emotionally relevant
123 sensory information into physiological and behavioral responses, project into the hypothalamus
124 (Gilpin et al., 2015). These projections dampen hypothalamic activity, such as autonomic anxiety
125 responses to fearful stimuli (Gilpin et al., 2015; Nuss, 2015). Decreased GABA activity is often
126 anxiogenic - that is, it induces anxiety (Nuss, 2015). Studies conducted in animals have shown
127 that administration of GABA receptor agonists into the amygdala, leads to a decrease in fear and
128 anxiety observed in these models (Nuss, 2015). Benzodiazepines, a class of antianxiety
129 medication, enhance the neuronal inhibitory action of GABA via allosteric effects at GABA
130 receptors, leading to enhanced anxiolytic (reduced anxiety) or tranquilized states (Bleakley &
131 Davies). Thus, several studies discussed later investigate differential DNA methylation or
132 histone modifications associated with genes associated with GABA and its receptors.

133 The amino acid glutamate is the major excitatory neurotransmitter in the mammalian
134 brain and CNS that counterbalances GABA's inhibitory actions (Nuss, 2015). This cooperation
135 can be seen when considering the previously mentioned GABAergic pathway starting in the

CeA. Before the CeA can exert its inhibitory effect on the hypothalamus, it receives glutamatergic or excitatory input from the basolateral amygdala (BLA), which has been implicated in Pavlovian learning and receives input from the parietal, cingulate and prefrontal cortices (Pessoa, 2010; Nuss, 2015). In addition to its role at synapses, glutamate is also partially responsible for neurogenesis, synaptogenesis and neurite outgrowth, similar to the neurotrophin BDNF. It is capable of binding to two types of receptors: NMDA and AMPA, which are gated Ca^{2+} and Na^{+}/K^{+} channels respectively (Riaza et al., 2011). It has been previously demonstrated that the inhibition of NMDA receptors at synapses blocks fear acquisition, and that acute stress appears to increase glutamate release as well as glutamate receptor expression, particularly the NMDA receptors which modulate the secretion of corticotropin-releasing factor/hormone (CRF/CRH), discussed later, in the CeA (Levenson & Sweatt, 2005; Riaza et al., 2011). Many antagonists of NMDARs and AMPARs in animal models have shown anxiolytic (reduced anxiety) outcomes, suggesting that these receptors may be possible pharmacological targets for treating ADs (Riaza et al., 2011).

Hormonal activity also plays a pivotal role in the anxiety response. The most well-studied governor of the stress response, the HPA axis, involves a negative feedback mechanism between the hypothalamus, the pituitary gland and the adrenal glands (Klengel et al., 2014). The paraventricular nucleus (PVN) of the hypothalamus secretes arginine vasopressin (AVP) and CRF into the hypophyseal portal system, a capillary bed that connects the hypothalamus to the anterior pituitary gland. Here, these neuroendocrine chemicals promote the production of adrenocorticotropic hormone (ACTH), which is released into the bloodstream. ACTH then binds to ACTH or melanocortin type 2 (MC2) receptors of the adrenal gland, which stimulates the release of corticosteroids from its cortex (Klengel et al., 2014). These corticosteroids are

commonly known as ‘stress hormones’, and are capable of binding to two intracellular receptors: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). Once bound to their hormone ligand, MRs and GRs act as transcription factors by migrating to the nucleus and binding to hormone response elements often found in gene promoters (Funder, 1997). Binding of corticosteroids to GRs in the pituitary and hippocampus inhibits the production of CRF, establishing a negative feedback loop (Fig. 3). In the adrenal glands, this binding promotes the release of epinephrine and norepinephrine, two hormones involved in the fight-or-flight response (Edwards & Guilliams, 2010; Bartlett et al., 2017).

Methodology

Animal Testing in Neuroepigenetics

Elucidating molecular changes in the human brain is made difficult by timing of sample acquisition, cell type distribution between samples, cause of death and brain agonal state, as well as sample handling, storage temperatures and sample size (Bakulski et al., 2016). Findings gleaned from post-mortem brains must be considered carefully, as undocumented confounding factors such as recreational drug use and non-prescribed medicine abuse may bias results. Additionally, degradation of epigenetic markers, particularly histone acetylation, has been observed with more time elapsed post-mortem (Jarmasz et al. 2019). Many epigenetic studies conducted in humans involve the retrieval of peripheral samples such as cord, systemic blood, or saliva (Schiele & Domschke, 2020). As such, much of neuroepigenetic psychiatric research relies on the use of animal models, particularly rodents.

Brain circuitry underlying anxiety, including neuronal pathways and neurotransmitters, is highly conserved between humans and rodents (Hohoff, 2009). Of course, replicating a

psychiatric disorder in animals with underdeveloped cortices compared to that of humans, poses some difficulty when attempting to study anxiety holistically. Symptoms of mental illnesses are accompanied by emotional, cognitive and motivational aspects that are not attributable to the lower mammal species being studied (Lezak et al., 2017). In 2009, the National Institute of Mental Health (NIMH) initiated the Research Domain Criteria (RDoC) project, with their stated goal as: "[to] transform the understanding and treatment of mental illnesses through basic and clinical research" (Cuthbert, 2015). As such, for different disorders, an accepted literature has emerged wherein several methods and tests have been accepted as standards for inducing/modeling and measuring anxiety-like or depressive-like symptoms and behaviors in animals, particularly in rodents. Of course, improper and inconsistent handling of rodents within a treatment group may skew any results obtained (Walf & Frye, 2007). One often runs the risk of over-interpreting data retrieved from animal studies when applying them to human psychiatric theory, since studying anxiety in animals requires a simplified, reductionist approach (Hohoff, 2009).

Modeling Anxiety

To measure anxiety-like behaviors in animals, researchers must first create an anxiety model by applying different stressors to the animal. There are also a variety of rodents available that have been bred to have lower or higher responses to stress, or with different innate anxiety temperaments, with relevant anxiety outcomes, several of which are discussed in this review (Simmons et al., 2012; Chaudhury et al., 2014; Sotnikov et al., 2014). It is important to understand that while these stress paradigms very rarely mimic those experienced by humans, they evoke a relevant anxiety phenotype in the animal model. Stressors typically include restraint in a cylindrical, perforated tube, application of an electric shock to the foot (0.5-2mA) for 1-2

seconds, maternal separation following birth, cage tilting, disrupted light-dark cycles and food deprivation, to list a few (Campos et al., 2013). Several stress paradigms that permit anxiety modeling may be employed as follows in **Table 1** (reviewed in Lezak, 2017).

Measuring Anxiety

Multiple tests are often employed within a single study to observe anxiety-like behaviors. These tests either measure an animal's avoidance behavior or defensive behavior, when a threat is perceived or introduced. Both of these behaviors imply anxiety-like phenotypes in these models. Furthermore, the use of rodent models in anxiety research has been consistently validated through the administration of drugs that exert anxiolytic and anxiogenic effects in humans, where they are shown to exert similar effects that are in turn, measurable according to the tests discussed below (Fig. 4) (Hohoff, 2009). Note that there are a plethora of tests that can be employed to measure anxiety, such as novelty-induced hyponeophagia, elevated zero mazes, Geller-Seifter and Vogel tests, and marble-burying, not discussed here (Harro, 2018). The three most commonly used tests in the studies included in this review are summarized below in **Table 2**.

Avoidance behaviors measured in these three assays are homologous to the maladaptive avoidance behaviors observed in human anxiety disorders, where perceived threats, such as places and situations, are avoided (Hohoff, 2009).

Additionally, applications of Pavlovian fear conditioning have led to the development of other assays that measure anxiety-like behaviors in animals. For example, pairing an aversive unconditioned stimuli such as a footshock, with a tone/sound, light or a context/environment (neutral conditioned stimuli), can elicit fear responses to the conditioned stimuli in the absence

of the footshock upon re-exposure to the conditioned stimulus (Lezak et al., 2017). **Acoustic startle** is a measurable 'flinch' to a delivered white noise, that has an amplitude that can be quantified in units of force, and is hypothesized to reflect the state of alertness associated with increased anxiety (Lezak et al., 2017). **Freezing behavior** is the time an animal spends immovable/frozen in fear upon application of the conditioned stimulus, and serves as a measure of anxiety-like behavior where behavioral inhibition is observed (Korte, 2001). Importantly, assays reliant on Pavlovian fear conditioning are also applicable to PTSD models, so results should be interpreted carefully (Hohoff, 2009).

Molecular Tests

Within the field of epigenetics, several specialized assays have emerged transforming the way we conduct research in the 21st century. These techniques often require homogenized tissue samples, such as centrifuged blood, or rodent brain samples.

Bisulfite Sequencing is used to identify methylated DNA (5mC) by converting all unmethylated cytosine bases to uracil through the addition of a bisulfate agent such as sodium bisulfate. This detection method does not work for identifying 5-hydroxymethlycytosine (5hmC). In a follow-up PCR, the uracil residues are converted to complement thymines, whilst the 5mC remains unconverted (Fig. 5) (Li & Tollefsbol, 2011). 5mC is then detectable by a subsequent RT-PCR step by use of methylation-specific primers (these vary per study) which will result in thousands of amplicons that can then be sequenced via next-generation sequencing or identified by whole genome methylation arrays (Yong et al., 2016). Methylation content can also be assessed as a percentage at CpG sites, as a ratio of cytosine to thymine (Li & Tollefsbol, 2011). Darst et al. have described the five basic steps in bisulfite conversion of DNA as follows: 1) denaturation of the DNA sample (~2µg genomic DNA); 2) addition and incubation with bisulfite

agent at elevated temperatures (98°C) for deamination (conversion of cytosine to uracil); 3) desalting to remove the bisulfate; 4) desulfonation of sulfonyl uracil adducts in the sample DNA which tend to form at alkaline pH; and 5) removal of the desulfonation solution used (Darst et al., 2010). Bisulfite sequencing provides a readout of the methylation status of every individual cytosine within a defined region of the genome, therefore permitting the identification of differentially methylated DNA between anxiety and control models as an example.

Chromatin Immunoprecipitation, also referred to as ChIP, is capable of detecting and mapping protein-DNA interactions, such as DNA-binding sites for specific proteins. These proteins include transcription factors and other chromatin modeling proteins such as DNMTs, but most importantly, they include histone modifications/markings (Park, 2009). ChIP may also be used to retrieve DNA with 5hmC marks (Papale et al., 2017). The first step of ChIP requires the temporary crosslinking of DNA with DNA-bound proteins using formaldehyde in the sample of interest. This ensures retrieval of the target DNA, and buffers against loss of chromatin bound to the protein of interest. The chromatin is then sheared by sonication, where the sample is exposed to ultrasonic frequencies/vibrations. Here, the DNA fragments are approximately 200-1000 base pairs long (Nelson et al., 2006). The subsequent success of ChIP relies on the validity and quality of specific antibodies chosen for the target protein being investigated, for example, anti-H3K27me. Antibodies are usually coupled to magnetic beads and immunoprecipitate with the histone mark being investigated by binding in a structure specific manner – the overall principle of ChIP. The beads are retrieved using magnets, or centrifugation, depending on the bead type used, and the sample isolate which now contains bead-antibody-protein-DNA target sequence complexes, is washed (Song et al., 2015). Any chromatin not bound to the target histone mark is ultimately lost when the target DNA is ‘pulled down’ via the antibody coated

beads. The protein-DNA is unlinked using proteinase K which removes the antibodies and the target protein, and the sample is centrifuged to purify the DNA (Nelson et al., 2006; Flanagin et al., 2008). The retrieved DNA can then be amplified via PCR, and/or analyzed by hybridizing these fragments to a microarray (ChIP-chip). With technological advancements however, pairing ChIP with next generation sequencing, commonly referred to as ChIP-seq, has proven to be far more beneficial for the purpose of epigenetic studies. Though ChIP-seq is more costly, it requires less DNA input (Park et al., 2009). Sequencing of the target DNA allows researchers to determine what genes are under the control of specific histone marks. Pairing ChIP-seq with Western blots and immunofluorescent or immunohistochemical assays which detect up- or downregulation of specific histone marks in a sample tissue allows researchers to formulate relationships between a differential histone mark and an observed change in gene expression.

Findings

Target genes and protein products of these neuroepigenetic studies in anxiety models are numerous and variable. **Table 3** below summarizes these targets and their abbreviations for more comprehensive reading.

A. DNA Methylation

DNA methylation plays crucial roles in gene silencing events, particularly at the promoter regions of genes, and varies per tissue type (Ohgane et al., 2008). Caution should be taken when comparing patterns of methylation as well as the resulting gene expression differences in peripheral samples to CNS samples of different brain regions, and when comparing different brain regions. Within the scope of DNA methylation studies, studies may focus on the presence of DNMTs, without elaborating on specific genes affected by any

abnormal levels of these enzymes measured, while other studies may focus on promoter methylated trends of genes implicated in AD pathophysiology, where the DNMTs responsible for differential gene expression patterns were not necessarily investigated.

DNMT Expression

Investigations into the patterns of DNA methyltransferases expression offer insight as to how these enzymes are able to respond to external stimuli in order to epigenetically modify gene expression in the CNS. Expression levels of DNMTs positively correlate to DNMT activity and thus to any global methylation trends observed (Slack et al., 1999). Whilst DNMT expression levels are important to investigate, failure to study the connection between their expression trends and methylation trends of anxiety related genes in different brain regions, makes pathophysiological conclusions difficult to formulate, though it leaves room for future research directions.

Recent studies have reported anxiety-like behaviors in adult mice following prenatal deletion of *Dnmt1* from neural stem cells. Further analysis to uncover the downstream effects of differential *Dnmt1* expression has not yet been conducted with regards to anxiety (Noguchi et al., 2016). In a previous study, Low Novelty-Responding (bLR) rats, bred to exhibit increased anxiety and depressive-like behaviors, displayed decreased mRNA levels of Dnmt1 in the dentate gyrus (DG) and CA3 of the hippocampus, compared to their High Novelty-Responding (bHR) counterparts (Simmons et al., 2012). This DG-CA3 circuitry is believed to be responsible for event sequence-related memory formation and fear learning which underlies the anxiety-phenotype (McEwen et al., 2011; Zhang et al., 2014). Overall, these studies indicate that decreased levels of Dnmt1 underlie the anxiety phenotype. It can be postulated that this may be a result of reduced global methylation.

Previously, significantly reduced expression of *DNMT3a* was detected in blood samples retrieved from an anxious cohort consisting of young adults that correlated directly with anxiety severity (Murphy et al., 2014). Individuals of the anxious cohort also displayed higher levels of global methylation compared to non-anxious individuals, though site specific methylation trends were not assessed (Murphy et al., 2014). In another study, C57BL/6J mice exposed to aggressive CD1 mice to induce chronic social defeat stress (CSDS), displayed selective downregulation of *Dnmt3a* in their mPFC (Elliott et al., 2016). This correlated with a significant reduction in global DNA methylation of the mPFC, contrary to the increased methylation observed in human blood samples. While an increase in blood methylation may serve as a biomarker for anxiety, this methylation increase may not necessarily be observed in the brain.

More specifically, Dnmt3a1, a splice variant of Dnmt3a, was significantly reduced in CSDS mice, with no relevant changes in Dnmt3a2 (Elliott et al., 2016). Most interestingly, the researchers found a negative glucocorticoid response element sequence upstream of *Dnmt3a1* TSS, which specifically binds to NR3C1, the gene that encodes for the glucocorticoid receptor (GR) which binds to the stress hormone cortisol in order to regulate HPA axis response, suggesting a possible pathway through which Dnmt3a may exert anxiolytic effects when expressed. (Elliott et al., 2016). Viral knockdown of *Dnmt3a* induced the same anxiety-like phenotype previously observed in CSDS mice as measured by the EPM test, while *Dnmt3a1* viral overexpression in mouse dorsal medial prefrontal cortex (mPFC) - which regulates fear, anxiety, risk taking, and decision making - rescued CSDS-induced anxiety (Chocyk et al., 2013; Elliott et al., 2016). This suggests that Dnmt3a in the mPFC plays a pivotal role in the development of anxiety. Specific genes that are regulated by Dnmt3a in the mPFC, and thus, how enzyme knockdown induces the anxiety phenotype, remain elusive. Ontological and

functional analysis of genes expressed at sites of differential Dnmt3a activity would help to further elucidate its specific role in the development of the anxiety phenotype.

A novel study comparing juvenile mice on low-methyl diet vs. normal diets, investigated the effects of methyl deficiency on DNA methylation and Dnmt expression. Interestingly, researchers found that mice lacking methyl donors displayed decreased expression levels of both Dnmt3a and Dnmt3b in the hippocampus, a finding that correlated with impairment in hippocampal fear memory acquisition and reduced anxiety-like behaviors, as well as a decrease in the expression of *Grin2b*, a glutamate receptor involved in excitatory pathways (Nuss, 2015; Ishii et al., 2014). Of note, there was a moderate increase in *Grin1* expression, later observed in rhesus monkeys discussed in the *Other Genes* section below. These expression levels were reversed upon administration of a normal diet, though anxiety-like behaviors became elevated. This particular study highlights the role of DNMTs in fear memory consolidation and plasticity in the hippocampus at younger ages, possibly forming the core psychopathology of inappropriate anxiety responses that may carry into adulthood (Ishii et al., 2014).

Lastly, knockdown of *Dnmt3a* in the mPFC of rats resulted in an anxiety-like phenotype in Elliott et al.'s study, contrary to the *reduced* anxiety behaviors observed following a decrease in *Dnmt3a* expression in the mouse hippocampus in Ishii et al.'s research. This suggests that even when DNMT expression patterns are similar, consequences of these trends vary per brain region when influencing the anxiety phenotype. We can hypothesize that the collection of genes regulated by Dnmt3a in the hippocampus, differ from those of the mPFC.

NR3C1 and FKBP5

The *NR3C1* gene is composed of multiple exons and codes for the glucocorticoid receptor (GR), which binds the hormone cortisol in a pathway that regulates the HPA axis response during stress. Of interest to researchers is the methylation status of this gene as well as its overall expression patterns that underscore the anxiety phenotype. Previous studies have shown that prenatal exposure to maternal depression and increased cortisol levels significantly increase methylation of the *NR3C1* gene in neonatal cord blood samples at exon 1F (Oberlander et al., 2008; Hompes et al., 2013). Data from the TRAILS (Tracking Adolescents' Individual Lives Survey) study in Dutch adolescents (mean age: 16 years) showed similar hypermethylation at exon 1F in whole blood samples of individuals who reported stressful life events (SLEs) in childhood and adolescence, including sexual abuse and other trauma (van der Knaap et al., 2014).

However, divergent research has shown hypomethylation of exon 1F in the promoter region of *NR3C1* in leukocyte blood samples from individuals (18-65 years) who experienced adverse childhood events. Additionally, individuals diagnosed with an AD who did not report adverse childhood events, showed a similar trend of reduced methylation (Tyrka et al., 2016). This suggests that early stressors in childhood may epigenetically poise an individual toward anxiety pathology, where a decrease in methylation correlates to an increase in GR expression and overall hyperactivity of the HPA axis (Tyrka et al., 2016). These human studies leave much to be desired, insofar as they do not address the anxiety phenotype, nor measure GR expression levels.

In consensus with increased methylation studies of *NR3C1*, hypermethylation of exon 1F at several CpG sites correlated with a decrease in the mRNA levels of GRα (one isoform of glucocorticoid receptor) in samples of peripheral blood mononuclear cells (PBMCs) of adults

diagnosed with generalized anxiety disorder (GAD) (Wang et al., 2017). Higher levels of serum cortisol were also detected in GAD individuals. Of note, >50% of the GAD group had comorbid depression and approximately 42% smoked (Wang et al., 2017). Wang et al. argue that Tyrka et al. failed to homogenize the population sample (e.g. by AD diagnosis) and that the use of childhood traumatic experiences (CTEs) as a criterion adds uncontrollable variability to the results, though arguably, Wang's study is also confounded by comorbid depression and substance use. Individuals in Wang et al.'s study who reported CTEs, demonstrated lower levels of methylation compared to non-CTE GAD individuals. These researchers argue that reduced GR expression due to *NR3C1* hypomethylation, promoted HPA axis hyperactivity and increased cortisol production as a result of decreased negative feedback regulated by GRs (Wang et al. 2017.)

In concordance with the hypomethylation hypothesis, however, N3RC1 heterozygote mice (*NR3C1*$^{+/-}$) with depleted levels of GRs, showed a significant increase in anxiety-like but not depressive-like behaviors. Additionally, hypomethylation of *FKBP5* which encodes FK506 binding protein 5, a proximal protein regulator of GRs that has been shown to decrease GR affinity for its ligand cortisol, therefore disrupting the negative feedback loop in the HPA axis, was reported in the placenta (Wochnik et al., 2005; Schmidt et al., 2019). Overexpression of *FKBP5* as a result of decreased *FKBP5* methylation in the amygdala is associated with the anxiety phenotype in adult rats (St-Cyr et al., 2017). Additionally, FKBP51 knockout mice are also more resilient to CSDS (Hartmann et al., 2012). In a follow up study, researchers virally overexpressed mutant FKBP51 in the BLA, which is involved in Pavlovian fear learning and receives sensory input from the parietal, cingulate and prefrontal cortices, elicited anxiety-like behaviors in mice (Fig. 6) (Pessoa, 2010; Hartmann et al., 2015). Treatment with Ligand2, an

antagonist specific to mutant FKBP51, had significant anxiolytic results in these mice, measured by the open field, EPM and light/dark box tests. Another inhibitor, SAFit2, which is capable of inhibiting wildtype FKBP51, also reduced anxiety-like behaviors 16h following administration in naïve adult mice following either peripheral or BLA-injected administration (Hartmann et al., 2015). These findings suggest that FKBP51 inhibitors may be used as a potential pharmaceutical intervention for anxiety across demographics.

In humans, decreased levels of *FKBP5* methylation detected in blood samples was found to be associated with better CBT treatment outcomes from pre- to post-treatment patients formally diagnosed with phobias. Meanwhile patients with no changes or increased levels of *FKBP5* methylation had poorer therapy outcomes in comparison (Roberts et al., 2017). Similar findings, with decreased *FKBP5* methylation detected in saliva samples associated with better CBT outcomes, were also reported in a cohort of children diagnosed with anxiety between the ages of 8-15 (Roberts et al., 2015). While these findings contradict data retrieved from St-Cyr et al.'s and Hartmann et al.'s rodent models, where decreased methylation and subsequent increase in FKBP5 expression underscored the anxiety phenotype, these studies suggest that *FKBP5* methylation levels in the blood and saliva can be used to determine populations that may benefit from aggressive CBT regimens.

Gamma-amino butyric acid (GABA)

GABA, the main inhibitory neurotransmitter of the CNS, is another much studied candidate gene in anxiety research. In newborns of pregnant mothers that experienced anxiety measured by PRAQ, a pregnancy-related anxiety questionnaire, researchers found that an increase in methylation of CpG islands of *GABBR1* in the cord blood of male newborns ($GABA_B$ receptor subunit 1 gene), was associated with higher anxiety levels of pregnant mothers as well

as increased cortisol levels in these infants upon vaccination (applied stressor) (Vangeel et al., 2017). Similar methylation trends were observed at the *NR3C1* gene in the previously discussed neonatal cord blood study further validating the impact of prenatal stressors *in utero* on the methylation status of genes in newborns (Oberlander et al., 2008; Hompes et al., 2013).

Studies of methylation trends pertaining to GABA-associated genes in animals permit us to further study the consequences of aberrant expression levels. In one study, researchers used H67D male mutant mice that contained increased levels of redox-active iron in the brain. They found that with an increase in brain iron load, global methylation, Dnmt1 mRNA levels and activity, were all decreased (Ye et al., 2018). Additionally H67D mutant mice with decreased *Dnmt1* expression exhibited lower levels of anxiety in the EPM assay compared to wildtype counterparts. However, these findings contradict Simmons et al. study in anxious bLR rats as well as Noguchi's prenatal *Dnmt1* deletion mice, where reduced *Dnmt1* expression led to an increase in the anxiety phenotype. The investigators found an increase in *Gabra2* ($GABA_A$ receptor subunit 2) mRNA levels by 140% in the mutant mice, as well as an overall decrease in GABA with a decrease in global methylation and *Dnmt1* expression and activity. Whether the increase in *Gabra2* expression is due to decreased methylation and reduced *Dnmt1* expression, or to the reduction in GABA, remains unclear (Ye et al., 2018). Binding sites for Dnmt1 on the *Gabra2* gene can be pursued in future studies.

Contradictorily, a study conducted in mice fed a methyl-deficient diet, showed the opposite effects, such that a decrease in the expression of *Gabra2* in mouse hippocampus correlated with a decrease in anxiety-like behaviors, though hippocampal and whole brain studies are difficult to compare. Furthermore, though *Dnmt3a* and *Dnmt3b* showed significant reduction in expression, these levels do not explain a decrease in Gabra2 levels, and DNMT1

453 levels were unaffected in low-methyl fed mice (Ishii et al., 2014). This suggests that *Gabra2* is
454 under the regulation of multiple epigenetic factors and not a single independently acting enzyme
455 like a DNMT.

456 While the Ye et al. iron study failed to investigate levels of *Dnmt3a* and *Dnmt3b*, Ishii et
457 al.'s failed to correlate decreases in *Gabra2* levels with detected GABA levels. As previously
458 mentioned, harmful prenatal exposures include endocrine disrupting chemicals (EDCs) such as
459 bisphenol-A (BPA). In one study using rat dams fed BPA, newborns showed an increase in
460 Dnmt 1 mRNA expression levels in their basolateral amygdala (BLA) in tandem with
461 increased anxiety behavior observations (Zhou et al., 2013). Researchers believed that the
462 GABAergic pathway was affected, because an increase in Dnmt1 correlated with a decrease in
463 glutamine decarboxylase (GAD67) mRNA, the enzyme responsible for the production of GABA
464 from glutamate, though GABA and glutamate levels were not directly measured (Valenzuela et
465 al., 2011; Zhou et al., 2013). This anxiogenic effect of increased Dnmt1 in the BLA parallels the
466 anxiolytic effect of decreased Dnmt1 in Ye et al.'s H67D mice. Researchers also showed that
467 reversal of decreased GAD67 mRNA expression and subsequent anxiety-like behaviors in BPA
468 rats is possible by administering 5-ada-Cdr, a hypomethylating agent (Zhou et al., 2013). The
469 rescuing effect of 5-ada-Cdr was then later inhibited by use of picrotoxin (PTX), an antagonist of
470 the $GABA_A$ receptors (Zhou et al., 2013). Taken together, these findings show a clear role for
471 decreased GABA in the creation of the anxiety phenotype, and that the multiple epigenetic
472 changes underscoring this decrease provide potential therapeutic targets when considering
473 treatments.

474 In a follow-up study published five years later, the activity of glutamatergic pathways in
475 relation to GABAergic inhibition, is somewhat elucidated in mice. Employing a prenatal restraint

stress (PRS) model on pregnant dams, researchers found that PRS offspring displayed similar anxiety-like behaviors as BPA-treated mice, as well as an increased binding of overexpressed Dnmt1 to the promoter region of *Gad67* (Fig. 7) (along with MeCP2, discussed later). This provided a direct relationship between the decreased mRNA levels of GAD67 (repressed by promoter hypermethylation) and overexpressed Dnmt1 (increased methylating activity), as well as underscoring a direct role of prenatal stressors *in utero* and later observed anxiety phenotypes in offspring (Zhu et al., 2018). Most interestingly, these researchers conducted electrophysiological analysis on brain slices of PRS and control mice and found that upon stimulation of the entorhinal cortex (EC), PRS mice displayed a greater number of population spikes (PSs) in the BLA. These findings were attributed to higher neuronal firing rates and cortical-BLA synaptic activity, suggesting that a decrease in GAD67 expression impairs GABAergic pathways, which in turn fail to inhibit glutamatergic or excitatory pathways in the BLA (Zhu et al., 2018). Manipulation of GAD67 expression by administering drugs that target the epigenetic markers regulating its expression, provides an exciting avenue for future treatment possibilities.

CRH/CRF Related Genes

The *CRH/CRF* gene codes for corticotropin releasing hormone/factor and therefore plays a pivotal role in the regulation of the HPA axis and stress responses involved in anxiety. Prolonged demethylation of the *Crf* promoter in adult mice that displayed social avoidance has been reported in a CSDS model, accompanied by a subsequent increase in *Crf* mRNA levels in the PVN of the hypothalamus of these animals. Both findings were significantly reversed upon administration of the antidepressant imipramine (Elliott et al, 2010). Researchers also detected decreased levels of *Dnmt3b*, but more interestingly, it was observed that the viral knockdown of

499 *Crf* in the PVN buffered against social avoidance behavior in CSDS mice. This suggests that
500 increased *Crf* expression may underscore increased social anxiety behaviors in these animals by
501 inducing HPA axis hyperactivity (Elliott et al, 2010).

502 Prenatal stress has also been shown to alter methylation states of *CRH* as previously
503 shown in *GABA* and *NR3C1* cord blood samples studies. New mothers exposed to war conditions
504 in the Democratic Republic of Congo exhibited different CRH methylation patterns based on the
505 type of stress reported: war stress or chronic stress, highlighting that the stress type experienced
506 influences the epigenetic change observed (Kertes et al., 2017).

507 Male rats born to dams subjected to PRS showed an increase in anxiety-like behaviors
508 upon assessment with open-field and EPM tests, as well as higher serum levels of corticosterone.
509 PRS offspring also showed higher corticosterone concentrations when subjected to their own
510 restraint stress session compared to the control group, indicating HPA axis hyperactivity in PRS
511 offspring (Xu et al., 2014). In the hypothalamus, CRH mRNA expression was decreased in the
512 PRS group before restraint stress administration, but increased significantly following the
513 restraint stress. This suggests that *in utero* exposure to elevated maternal corticosterone
514 concentrations epigenetically primed PRS offspring for later *Crh* overexpression when exposed
515 to stress (Xu et al., 2014). These findings suggest that before birth, an individual may already be
516 more at risk of developing an AD based on the mother's physical and mental state during the
517 pregnancy. PRS offspring also exhibited decreased *Crh* promoter methylation in the
518 hypothalamus compared to control animals, suggesting that the recorded increase in
519 corticosterone is a result of HPA axis hyperactivity and failure to decrease *Crh* expression
520 through the negative feedback loop (Xu et al., 2014).

Another prenatal stressor, gestational hypoxia (GIH), induced anxiety-like behaviors in newborn male rats. In the hypothalamic PVN, an increase in CRH and CRHR1 (a CRH receptor gene) was observed in male offspring, but not females, suggesting a sex-related positive stress adaptation in female animals (Wang et al., 2013). In both 19-day old male embryos and 90-day old male GIH offspring, hypomethylation of CpG islands within the *Crhr1* promoter were observed, suggesting that the hypomethylation of *Crhr1* initiated *in utero* persists even after birth into adulthood (Wang et al., 2013). Similar findings were observed in peripheral blood samples of patients diagnosed specifically with panic disorder, making *Crhr1* hypomethylation a possible diagnostic marker for panic disorder and other ADs (Schartner et al., 2017). To better understand this trend of hypomethylation, Wang et al. reported that while *Dnmt1* and *Dnmt3a* were unaltered in male and female GIH embryos, contrary to aforementioned anxiety studies (though different stress paradigms and brain regions were used), DNMT3B was downregulated in male embryos and upregulated in female embryos, possibly explaining the methylation differences of *Crhr1* in male and female offspring. The decreased expression of DNMT3B in the PVN persisted into adulthood in 90-day old male GIH rats (Fig. 8) (Wang et al., 2013). Both Wang et al.'s and Elliott et al.'s CRH/Crf studies show a direct correlation between Dnmt3b levels and CRF-associated genes in the PVN, suggesting that in this brain region, Dnmt3b may be responsible for modulating their expression via methylation. The effect of *Dnmt3b* knockout in PVN cells on the methylation state of CpG sites in the *Crhr1* promoter would be an interesting future study. We can hypothesize that a decrease in methylation of the *Crhr1* promoter leads to overexpression of *Crhr1* and overall HPA axis hyperactivity. ChIP-seq can be used to elucidate binding sites of Dnmt3b to CpG sites in the promoters of *Crf* and CRF-related genes since there appears to be a strong link between decreased *Dnmt3b* and *Crf* expression. In another study using

high-anxiety behavior (HAB) and low-anxiety behavior (LAB) mice, bred for innate levels of anxious demeanors (not to be confused with bHR/bLR animals), researchers reported that not only do HAB mice display an overexpression of CRHR1 (CRH receptor) in the basal amygdala compared to LAB mice, but that exposure of HAB mice to an enriched environment (EE) positively stimulates the animal, reversing this expression. Additionally, exposure of LAB mice to chronic mild stress (CMS) shifted CRHR1 expression levels to that of anxious HAB mice (Sotnikov et al., 2014). These preliminary findings demonstrate a direct impact of the external environment on the anxiety-phenotype by modulating gene expression. The use of a CRHR antagonist in HAB mice had an anxiolytic effect, further supporting the role of overexpressed Crhr in various brain regions in observable anxiety phenotypes (Sotnikov et al., 2014). Interestingly, EE in HAB mice and CMS in LAB mice both caused an increase in methylation at CpG1 upstream of the *Crhr1* promoter in the amygdala. However, recall that in Wang et al.'s study, hypomethylation of this gene region in the hypothalamus (not amygdala) promoted an anxiety-like phenotype. In Sotnikov et al.'s study hypermethylation of CpG1 in the *Crhr* promoter can increase, or decrease, anxiety-like behaviors, emphasizing that gene expression regulation is incredibly complex as other epigenetic modifications may cooperate to regulate gene expression, which we will see below.

In an attempt to explain how a unidirectional epigenetic alteration can underscore a bidirectional shift in anxiety phenotypes, the researchers honed in on a transcription factor Ying-Yang1, or YY1, which was reported to be bidirectionally expressed between HAB-EE and LAB-CMS mice (Sotnikov et al., 2014). YY1 was found to bind near CpG1 of *Crhr1*'s promoter in response to methylation of CpG1. In HAB-EE mice, lower levels of YY1 expression coupled with an increase in methylation correlated with a downregulation of Crhr1 expression. An

increase in YY1 in LAB-CMS with an increase in methylation, correlated with the upregulation of Crhr1 expression (Sotnikov et al., 2014). Expression of YY1 may be attributed to EE (downregulation) and CMS (upregulation) as positive and negative stressors, respectively. Overexpression of YY1 in mouse neuroblastoma cells (N2a) was shown to enhance CRHR expression by increased promoter activity, suggesting that increased methylation at CpG1 of *Crhr1* in LAB-CMS anxiety-exhibiting mice does not repress CRHR expression when YY1 expression is increased (Sotnikov et al., 2014). Identification of this pattern of differential methylation of the *Crhr1* gene highlights brain plasticity, such that external stimuli such as EE and CMS, are able to alter gene methylation states and subsequently induce changes in anxiety-like behaviors via chromatin remodeling protein recruitment (Sotnikov et al., 2014). Whether YY1 recruits histone modifying complexes to the *Crhr1* gene in these models would be an interesting follow up study, as well as elucidation of any protein-protein interactions between YY1 and DNMT3B.

To summarize, though there is consensus that overexpression of CRH/CRF related-genes in the amygdala and hypothalamus underscores HPA axis hyperactivity and therefore contributes to the observed anxiety phenotype, elucidation of the epigenetic regulation of these genes is crucial towards pinning down anxiety-specific targets for therapy. Findings such as differential methylation trends and the expression levels of YY1 and Dmnt3b, highlight the need to pool existing literature to encourage more cohesive and expansive studies within the field.

BDNF

BDNF contains several functional exons, and its expression levels, transcripts and associated epigenetic markers vary per brain region by use of alternative splicing and promoters (Cattaneo et al., 2016). This makes it exceedingly difficult to characterize into simple,

straightforward trends when studying psychopathologies. Recall that BDNF is a neurotrophic factor that facilitates neurogenesis globally in the CNS (Cattaneo et al., 2016). We can therefore surmise that BDNF expression is beneficial based on the brain region and the timing of plasticity changes due to this neurogenesis, such as during periods of fear learning versus positive safety appraisal events. Several studies agree that BDNF serum levels fail to act as biomarkers for anxiety since dysregulated expression of this factor is present in multiple psychiatric illnesses including depression and schizophrenia (Molendijk et al., 2011; Carlino et al., 2015; Cattaneo et al., 2016). However, understanding the impact of DNA methylation states of *BDNF* is still crucial for understanding *BDNF* expression modulating the underlying neurophysiology of psychiatric illnesses. Like CRH, GABA and NR3C1, prenatal factors such as maternal depression may impact BDNF levels in the cord blood of neonates such that BDNF concentrations were significantly lower compared to healthy controls (Sonmez et al., 2016). The actual impact of decreased BDNF in these newborns can only be unearthed by follow-up studies that track these children over their lifespan. Given BDNF's role in brain plasticity, memory and learning, we can conclude that levels of BDNF at different developmental stages may influence resilience and vulnerability to anxiety during those developmental stages. A recent review by Poon et al., summarizes the potential in targeting BDNF expression with antidepressants to facilitate fear memory extinction in a depression paradigm that may also be used to alleviate anxiety-related pathology (2021).

Other Genes

Though rarely conducted, a single anxiety study on 23 rhesus macaque monkeys (~1.3 years old) matched phenotypically for anxious temperament (AT), analyzed genome-wide DNA methylation and mRNA expression in the CeA (Alisch et al., 2014). Primates such as these are

613 the best model for investigating relevant anxiety traits in humans, since they share genetic,
614 neuronal and phenotypic foundations of complex socio-emotional behaviors demonstrated by
615 humans. DNA methylation analysis of these AT monkeys revealed genome-wide
616 hypomethylation of CpG islands in promoter regions, particularly in transcription start sites
617 (TSS) (<10%), with higher methylation levels further away from the TSSs (Alisch et al., 2014).
618 Researchers also identified almost 5500 CpG sites with AT-associated methylation, 87% of
619 which showed a decrease in methylation levels with an increase in AT severity. Further analysis
620 revealed that AT-associated methylation events were more prominent in the gene body (55%)
621 with very little representation at the gene promoter (0-2%) (Alisch et al., 2014). Gene ontological
622 and functional analysis of AT-associated loci and changes in gene expression, included most
623 notably *GRIN1* and *GRM5*, which code for specific subunits of different glutamate receptors.
624 The genes *JAG1* and *BCL11A showed* methylation patterns that predicted gene expression in the
625 AT phenotype. *BCL11A* codes for a protein involved downstream of a glutamate receptor
626 cascade for dendritic arborization, such that a decrease in its expression resulted in extreme AT
627 (Alisch et al., 2014). *JAG1* on the other hand, acts as a ligand in NOTCH signaling, which plays
628 a critical role in CNS development, including synaptic plasticity and memory formation. An
629 increase in methylation was associated with a decrease in *JAG1* expression and an increase in
630 AT severity (Alisch et al., 2014). Comparatively, differential DNA methylation of *JAG2* was
631 reported in blood samples of twins (Alisch et al., 2017). *DMNT* expression levels were not
632 investigated in the macaque study, so the enzymes and mechanisms responsible for the reported
633 differential DNA methylation regions in the CeA were not defined.

634 Overall, there are a plethora of genes involved in the creation and persistence of the
635 anxiety phenotype. For example, serotonin-associated genes, such as receptor genes including *5-*

HTT, have been thoroughly reviewed within the scope of mood disorders by Klaus-Peter Lesch in *When the Serotonin Transporter Gene Meets Adversity: The Contribution of Animal Models to Understanding Epigenetic Mechanisms in Affective Disorders and Resilience* (2011).

Though a few major genes were discussed above in detail, as these are more popularly investigated, it is important to remember that no single isolated gene, nor individual epigenetic modification causes AD. Genome-wide studies, such as Alisch et al.'s, not only provide a great overview of the complex gene patterns that underscore anxiety, but also generate a list of potential novel gene targets, especially those associated with excitatory and inhibitory CNS signaling pathways, for future studies.

The DNA methylation patterns discussed in the above sections are summarized in **Table 4** below:

MeCP2: Bridging DNA Methylation and Histone Modifications

MeCP2, methyl CpG binding protein, is known to act as a bridge between DNA methylation marks and HDACs, enzymes involved in histone modifications and has been shown to dock at CpG methylated sites and recruit HDACs to the chromatin, forming a silencing complex that represses gene transcription (Fig.9) (Martinowich et al., 2003). This binding is partially impaired if CpG sites are hypomethylated (Martinowich et al., 2003). Following depolarization of mouse E14 cortical cells, MeCP2 was found to be partially dissociated from the *Bdnf* promoter, suggesting that neuronal firing may redistribute MeCP2 binding allowing for BDNF expression (Martinowich et al., 2003). In a study published a few years later, viral deletion of MeCP2 in the basolateral amygdala (BLA) of mice resulted in an increase in anxiety-like behaviors. This was accompanied by an increase in histone 3 (H3) acetylation, indicating

that MeCP2 may be required to recruit HDACs to maintain lower levels of acetylation necessary for gene repression (Adachi et al., 2009). This finding was further supported upon inhibiting HDACs with Trichostatin A (TSA), which resulted in reduced postsynaptic excitatory firing of cortical pyramidal neurons, a finding which paralleled Adachi et al.'s MeCP2 null cells. Overall, it appears that MeCP2 is able to modulate synaptic transmission frequency through transcriptional repression via HDAC recruitment (Kavalali et al., 2011).

Histone Modifications

Histone modifications within the CNS play key roles in both memory formation and consolidation as seen in the animal studies discussed below (Day & Sweatt, 2011). Most anxiety-based studies hone in on histone acetylation and methylation marks pertaining to differential gene expression, though other histone markers exist, such as phosphorylation, ubiquitylation, serotonylation and dopaminylation, of which the latter two are fairly new in the field of epigenetics, and have yet to be explored in any anxiety study (Berger, 2002; Farrelly et al., 2019; Lepack et al., 2020). HDACs, the enzymes responsible for histone deacetylation, are usually associated with transcriptionally silent chromatin and are often the easiest to study as numerous HDAC inhibitors are available for use in anxiety animal models (de Ruijter et al., 2003). As we discuss differential histone modifications below, it's important to remember that any observed changes can impact multiple transcripts at once.

Histone Marks and Gene Regulation

In a study using footshock paired with white noise in C57BL/6 mice (a model for PTSD and anxiety), animals that displayed fear extinction where the induced fear behavior such as freezing is lost by extinction training, had increased levels of histone H4 acetylation around the

promoter of *Bdnf* exon IV, compared to both naïve and fear-conditioned animals without-extinction controls. This hyperacetylation was concomitant with an increase in BDNF exon IV mRNA in the PFC of mice that achieved fear extinction (Bredy et al., 2007). It is likely that an increase in BDNF expression facilitated by H4 hyperacetylation underscores improved learning and subsequent extinction of fear-conditioned behaviors. Valproic acid (VPA), a HDAC inhibitor and mood stabilizer, was shown to potentiate long term memory for fear extinction, suggesting that HDACs may perpetuate repressed *Bdnf* expression in anxiety models (Bredy et al., 2007).

In another BDNF-focused study, researchers reported a decrease in repressive H3K9me2 at the promoter of exon IV in the hippocampus of male rats exposed to maternal separation (ES) following birth, which persisted for 2 months. This was accompanied by an observable increase in neurogenesis in the hippocampal dentate gyrus (DG) and improved performance on the Morris Water Maze stress-associated test, indicating better spatial learning propensities (Suri et al., 2012). The opposite is observed in adulthood at 15 months, where increased H3K9me2 at the *Bdnf* IV promoter, decreased hippocampal expression of *Bdnf IV* and subsequent neurogenesis were observed in ES animals (Suri et al., 2012). These findings suggest that there is an inverse relationship with H3K9me2 and BDNF. The researchers believed that increased *Bdnf* expression in early life may facilitate fear learning and avoidance behaviors, whilst reduced expression and brain plasticity in later life may impair fear extinction (Suri et al., 2012). The biphasic changes in hippocampal plasticity, memory and learning impairments observed in middle-aged animals appears to support the conclusion that while some epigenetic alterations may prove to be potentially adaptive in early developmental ages, shifts in histone methylation may have deleterious outcomes later in life (Suri et al., 2012). The antidepressant amitriptyline was able to

attenuate H3K9me2 increase and subsequent *Bdnf* IV expression decrease, as well as accompanying cognitive decline, suggesting that these drugs may bolster cognitive-behavioral therapy geared towards fear extinction learning (Suri et al., 2012). Other stress models have shown similar histone methylation trends in the hippocampal DG, such as in rats subjected to chronic restraint stress (Hunter et al., 2009). Reduced levels of H3K9me3 was detected, a finding that was reversed upon administration of the antidepressant fluoxetine, though neither gene expression changes nor anxiety phenotype were measured (Hunter et al., 2009). Conversely, though decreased levels of H3K9me and H3K9me3 were also observed in young female rats exposed to early maternal separation, these changes were observed in the PFC, and underscored reduced fear-startle (low anxiety phenotype) (Kao et al., 2012). This suggests that though the same expression of histone marks are observed, these marks may govern different transcripts depending on the brain region. Additionally, sex differences should also be taken into consideration as it has been reported that maternal separation tends to illicit higher anxiety-like behaviors in male offspring compared to females (Lehmann et al., 1999).

Lastly, elevated levels of H3K9me3 associated with the glucocorticoid receptor (GR) promoter was detected in the amygdala and hippocampus of bHR rats (low anxiety) compared to their anxious bLR counterparts. This increase was concomitant with a decrease in GR expression, suggesting that H3K9me3 suppresses GR expression in these brain regions in a manner that attenuates HPA Axis hyperactivity – a factor underscoring the anxiety response (Chaudhury et al., 2014).

Overall, these studies elucidate specific histone marks associated with unique gene expression patterns that underlie the anxiety models utilized. In order to deduce the upstream

regulation of these histone marks, other studies have investigated the histone-modifying enzymes responsible for these trends.

Enzymatic Regulation of Histone Modifications

The question that remains is: what enzyme manages these observed H3K9 methylation states? The HMT complex G9a/GLP, composed of the HMT euchromatic histone-lysine N-methyltransferase 2 (EHMT2) or G9a and G9a-like protein (GLP or EHMT1), has been shown to be involved in the mono- and dimethylation of H3K9 (Schaefer et al., 2009). Researchers have reported that postnatal knockout of G9a reduced the anxiety phenotype in mice, whilst mutation or deletion of one copy of the GLP gene in humans leads to Kleefstra syndrome, characterized by social behavior impairment, impulsivity, aggression and mental retardation (Schaefer et al., 2009). Thus, G9a/GLP seems to exert temporal effects on developmental histone methylation. In concordance with this hypothesis, researchers found that administration of UNC0642 or A-366, selective G9a/GLP inhibitors, had different anxiety outcomes depending on the age of drug reception. Embryos that were exposed to the drug *in utero* after pregnant dams received intraperitoneal injections of UNC0642, showed an increase in anxiety-like behaviors, whilst adult mice receiving either drug showed a significant reduction in the anxiety phenotype in a dose-dependent manner (Wang et al., 2018). Western blots conducted on whole brain extracts of adult mice treated with either drug showed the expected decrease in H3K9me2, though adult mice exposed to UNC0642 during gestation showed no changes in the level of H3K9me2, suggesting that the effect of G9a/GLP is developmentally sensitive (Wang et al., 2018). The researchers believed that the observed reduction in anxiety in adult mice models make both UNC0642 and A-366 potential therapeutic options for treating anxiety. Though no specific brain region nor specific gene such as BDNF, was investigated in this study, the correlation of reduced

global H3K9me2 and the observed decrease in anxiety-like behaviors, aligns with Suri et al.'s 2012 study, where an increase in repressive H3K9me2 led to decreased hippocampal neurogenesis and overall cognitive decline. This provides a potential therapeutic target - the G9a/GLP enzyme complex.

In 2013, Suri et al. published a follow-up paper to the maternal separation early stress (ES) study that reported biphasic responses to an increase in hippocampal H3K9me2. While young adult rats showed an increase in BDNF expression, they also demonstrated high-anxiety behaviors in the open field test compared to controls. This was not observed in middle-aged ES and control animals (Suri et al., 2013). While BDNF upregulation appears to underlie improved spatial learning in young adult mice, it also seems to promote the development of the anxiety phenotype not observed in middle-aged ES animals. In addition to BDNF, young adult and middle-aged animals expressed distinct transcriptomes with very little overlap (Suri et al., 2013). Curiously, in young adult rats with anxiety-like behaviors, the genes *Grin1* and *Grik2*, genes which code for subunits of different glutamate receptors, were differentially regulated compared to same age controls. ChIP analysis of histone modifying enzymes in these animals showed a biphasic expression of HDAC2 and HDAC8, such that young ES animals expressed reduced levels of these deacetylases compared to their middle-aged ES counterparts (Suri et al., 2013). Histone methyltransferase expression was also found to be altered, such that HMT Suv39h1 was downregulated in young adult ES rats, and significantly upregulated in middle-aged ES rats. The differential expression of histone modifying enzymes did not, however, translate into global acetylation and methylation changes in the brain, suggesting that differential anxiety-relevant histone modifications govern transcriptomes specific to each brain region (Suri et al., 2013). This follow up study shows that multiple cooperating histone modifying enzymes, such as HDACs

and HMTs, modulate the anxiety transcriptome, though further investigation of gene clusters under the regulation of these enzymes would help to formulate a bigger picture.

Histone Modifications Associated with the HPA Axis

Previous studies have shown that increased levels of corticosterone delivery to the amygdala (CeA) increases CRF mRNA, enhancing anxiety-like behavior as well as dysregulation of the HPA Axis (Shepard et al., 2003). In a rat model, delivery of increased concentrations of corticosteroids (CORT) was shown to induce chronic anxiety. Decreased H3K9ac was observed in animals infused with CORT compared to vehicle controls upon staining of CeA slices (Tran et al., 2014). Paralleling this finding, it has previously been reported that in a chronic variable stress rat model, H3K9ac and H4K12ac were decreased in the CA3 region of the hippocampus and DG (Ferland & Schrader, 2011). Though brain regions vary between studies, both studies suggest that a decrease in the H3K9ac marker is influenced by stressors, and may underscore the anxiety phenotype.

H3K9ac has previously been shown to regulate the promoter of the glucocorticoid receptor (Zhang et al., 2013). This supports the finding that CORT-infused animals expressed lower levels of glucocorticoid receptors, due to the decrease in H3K9ac reported, with a 5-7 fold increase in Crf mRNA expression levels in the Tran et al. study (2014). ChIP of H3K9ac revealed a significant decrease in acetylation at the GR promoter, emphasizing H3K9ac's role as a permissive mark for GR expression (Tran et al., 2014). GR-ChIP-seq revealed that CORT administration reduced GR sequestering of transcription factor AP-1, an interaction that would have suppressed CRF expression via the negative feedback loop in the HPA axis. Instead, increased AP-1 mediated CRF expression is observed in CORT animals (Tran et al., 2014). Further investigation through antibody binding revealed co-localization of the HDAC Sirtuin 6

(SIRT6) with GR, such that an increase in SIRT6 correlated with a decrease in GR expression. TSA, a previously mentioned HDAC inhibitor of Class I and II HDACs, was found to significantly increase H3K9ac and GR expression, whilst decreasing CRF mRNA levels in the CeA. This was accompanied by rescue of the anxiety phenotype and a reduction in SIRT6 (Tran et al., 2014). The researchers summarize the mechanism as follows: increased CORT delivery activates GRs which localize to the nucleus to suppress CRF production via AP-1 interaction. SIRT6 is recruited to the GR promoter where it deacetylates H3K9, reducing GR expression. This alleviates the suppression of AP-1 by GR, and CRF expression is sustained at an increased rate (Tran et al., 2014). Studies such as these attempt to hone in on candidate therapeutic targets, such as SIRT6. In HAB mice bred for high anxiety, hypoacetylation of H3 was also observed in the cingulate cortex, adding to the growing literature that suggests a decrease in histone acetylation marks in a variety of brain regions, tend to underscore anxiety phenotypes. Treatment with the HDAC inhibitor MS-275 (Entinostat) was able to rescue these lower acetylation trends while exerting an anxiolytic effect (Sah et al., 2019). Overall, these studies suggest that there is a potential niche for HDAC inhibitors in the treatment of anxiety phenotypes (Sah et al., 2019).

Substance Use Comorbidity

ADs, such as GAD and social anxiety, are often comorbid with substance use disorders, such as abuse of cocaine and alcohol (Noyes, 2001; Buckner et al., 2007). Comorbidity incidence is often high because the same brain regions are implicated in both disorders, such as the nucleus accumbens (NAc). The NAc is the reward center of the brain that consists of many types of GABAergic neurons, and is often implicated in cocaine use and addiction (Feng it al., 2014). Cocaine has been previously shown to induce a drug-specific transcriptome consisting of thousands of gene expression changes via differential histone modifications in the NAc (Feng it

al., 2014). In an earlier study, severe downregulation of HDAC5 in the NAc was observed in mice exposed to chronic cocaine usage, as well as mice exposed to chronic social defeat stress (CSDS), via their own unique mechanisms (Renthal et al., 2007). Treatment of CSDS mice with the antidepressant imipramine, partially restored HDAC5 mRNA levels to near basal levels (Renthal et al., 2007). Furthermore, *Hdac5* knockout mice developed more severe social avoidance behaviors following subjection to CSDS compared to wildtype counterparts (Renthal et al., 2007). Though the researchers investigated genes under the regulation of HDAC5 in cocaine-treated mice, they did not do so in CSDS mice (Renthal et al., 2007). It would be interesting to see if there is any overlap in differential gene expression between these two models.

Another cocaine study conducted in rats showed that viral overexpression of G9a, one of the protein components of the HMT complex G9a/GLP previously discussed, resulted in an increase in H3K9me2, and accompanied by an increase in rat sensitivity to cocaine and anxiety-like behavior (Anderson et al., 2018). These findings appear to align with those of Wang et al.'s study, where a decrease in G9a was concomitant with a decrease in H3K9me2 in low-anxiety animals.

Recall that a decrease in H3K9ac in the CeA was observed in correlation to the anxiety phenotype in animals infused with CORT (Tran et al., 2014). In another study, P rats bred for selective alcohol preference, have been shown to have higher *Hdac2* expression levels and a concomitant decrease in H3K9ac accompanied by anxiety-like behaviors (Moonat et al., 2013). Upon acute exposure to ethanol, P rats were found to have reduced expression levels of HDAC2, increased levels of H3K9ac at *Bdnf* exon IV promoter, and a diminished anxiety phenotype (Moonat et al., 2013). Selective inhibition of *Hdac2* by siRNA infusion also resulted in

840 decreased HDAC2 mRNA and a concordant increase in H3K9ac, strengthening the supposition
841 that HDAC2 modulates H3K9ac levels. An increase in dendritic spine density was observed in
842 the CeA only, for both acute ethanol exposure and siRNA infusion (Moonat et al., 2013).
843 Though the mechanism for HDAC2 inhibition by ethanol is unclear, the anxiolytic effect of
844 alcohol in social situations can be inferred from this model, as it appears that alcohol
845 consumption attenuates anxiety symptoms and phenotypes on an epigenetic level. In support of
846 this hypothesis, P rats consumed significantly less alcohol upon siRNA treatment and subsequent
847 HDAC2 inhibition (Moonat et al., 2013).

848 Studies such as these emphasize that not only do epigenetic modifications regulate gene
849 expression changes and have global CNS consequences that may render an individual susceptible
850 to a multitude of psychiatric disorders, but that these differential changes may also sustain
851 underlying psychopathologies in comorbid cases. The differential histone modifications
852 discussed above are summarized in **Table 5** below.

853 **Pharmacological Implications: Possible Epigenetic Rescue by HDAC Inhibitors**

854 More recent anxiety therapies have focused on the use of HDAC inhibitors. For example,
855 Valproic acid (VPA), a mood stabilizer, has been shown to significantly increase H4 acetylation
856 at B*dnf* exon IV promoter as well as its mRNA in the PFC of fear conditioned mice, when
857 administered prior to extinction training (Bredy et al., 2007). These findings accompany the
858 observation that VPA-treated mice that previously showed no fear extinction, had enhanced
859 long-term memory that supported the extinction of conditioned fear (Bredy et al., 2007; Whittle
860 et al., 2012). VPA has also been shown to restore previously reduced levels of H3K9
861 methylation, suggesting that the expression of histone methylating enzymes may be regulated by
862 specific histone acetylation marks (Kao et al., 2012). The administration of VPA prior to

extinction training suggests that VPA or VPA derivatives may be beneficial pharmacological interventions that can be used alongside CBT to improve learning outcomes of new, adaptive behaviors (Gavin et al., 2011), as VPA may encourage synaptic plasticity through enhanced *BDNF* expression (Fig. 10) (Bredy et al., 2007).

Other HDAC inhibitors that have been shown to restore histone acetylation whilst rescuing from the anxiety phenotype in animals include Trichostatin A (Tran et al., 2014), Vorinostat (Fujita et al., 2012) and MS-275 (Sah et al., 2019). MS-275 did not rescue from deficient extinction acquisition in a S1 mouse model that show no extinction learning (Whittle et al., 2012), whilst Vorinostat appeared to increase the expression of *Nr2b* (NMDA receptor gene) in the hippocampus via increased H3 and H4 acetylation at its promoter, a mechanism believed to facilitate fear extinction in rats (Fujita et al., 2012). This suggests that not only do HDAC inhibitors have varying degrees and mechanisms of effectiveness, but that the time of administration, such as before or during the application of a stressor versus after fear learning when the stressor has been removed, is crucial for its efficacy (Gavin et al., 2011).

Summary of Findings

In summary, significant differential DNA methylation and histone modifications have been reported between anxiety and healthy controls, impacting a plethora of genes, the abbreviations of which are listed in **Table 1**. Many studies suggest that exposure to a number of stressors *in utero*, such as maternal hormones released during stress (Xu et al., 2014; Kertes et al., 2017; St-Cyr et al. 2017), depression or anxiety (Vangeel et al., 2017; Zhu et al., 2018), endocrine disruptors (Zhou et al., 2013), low oxygen levels (Wang et al., 2013) as well as medications, may alter DNA methylation patterns in offspring, rendering them either resilient or vulnerable towards developing an AD.

As established, DNA methylation is modulated by enzymes known as DNMTs (Morris et al., 2016). Decreased expression of *Dnmt3a* (Murphy et al., 2014; Elliott et al., 2016) and *Dnmt3b* (Elliot et al., 2010; Wang et al., 2013; Ishii et al., 2014) has been reported on multiple occasions in both anxious human and animal models, as well as in both peripheral and CNS tissue samples. In particular, reduced expression of *Dnmt3b* appears to accompany *Crh*/*Crf* expression increases observed in HPA axis hyperactivity and the anxiety phenotype, suggesting that Dnmt3b is responsible for suppressing *Crh*/*Crf* via repressive methylation at its promoter region (Elliot et al., 2010; Wang et al., 2013). Likewise, overexpression of the CRF receptor, CRHR, has also been reported on numerous occasions in anxiety models, further emphasizing that aberrant HPA axis regulation underscores this phenotype (Wang et al., 2013; Sotnikov et al., 2014). Increased methylation of *Nr3c1*, the glucocorticoid receptor gene, has been reported more often than not, in anxiety models (van der Knaap et al., 2014; Wang et al., 2017).

Decrease in GR expression may damper the negative feedback mechanism in HPA axis function, leading to hyperactivity and the observed anxiety phenotype (Tran et al., 2014; Wang et al., 2017; Schmidt et al., 2019). The histone mark H3K9ac has also been observed in a chronic anxiety model, such that a decrease in H3K9ac resulted in decreased GR expression and increased CRF production (Tran et al., 2014).

Differential methylation trends of GABA- and glutamate-associated genes have also been discussed. Although up- or downregulation of these genes have variable impacts on the anxiety phenotype depending on the age of the animals, brain regions involved and type of stress used to induce the phenotype. Notably, DNMT1 appears to regulate GABA production through *Gad67* expression, such that an increase in DNMT1 is concomitant with decreased GAD67

transcription, possibly through increased methylation. Subsequent decrease in GABA is observed (Zhou et al., 2013; Zhu et al., 2018).

As it is most crucial to brain function and psychiatric disorders, *Bdnf* expression has been studied in detail. This gene provides a great model for illustrating the principle that epigenetic modifications are intricately tied together, such that a variety of markers cooperate to either silence or activate genes. In other words, no single epigenetic alteration acts independently. Methylation marks on the *Bdnf* promoter are bound by MeCP2, a methyl CpG binding protein that appears to recruit HDACs (Martinowich et al., 2003). These HDACs, particularly HDAC2 (Moonat et al., 2013) appear to silence *Bdnf* expression by removing permissive acetyl marks on associated histones such as H3K9ac, rendering the chromatin less available for active transcription of BDNF. As a result, reduced *Bdnf* expression can be rescued with the administration of HDAC inhibitors (Bredy et al., 2007), as well as acute ethanol exposure (Moonat et al., 2013), a finding that possibly explains the anxiolytic effects of alcohol consumption. Increased H4 acetylation has also been reported with concomitant *Bdnf* IV expression (Bredy et al., 2007). Histone acetylation marks are not the only histone modifications found to be associated with BDNF, however. Decrease in repressive H3K9me2 at the promoter of *Bdnf* exon IV has also been associated with increased *Bdnf* expression, and an increase in H3K9me2 has been shown to decrease this expression, highlighting a direct inverse relationship between the mark and *Bdnf* expression (Fig. 11) (Suri et al., 2012). Additionally, the histone methyltransferase G9a/GLP appears to be responsible for the presence of H3K9me2 such that an increase in G9a is concomitant with an increase in H3K9me2 and a decrease in G9a/GLP is observed when decreased levels of H3K9me2 are detected (Anderson et al., 2018; Wang et al., 2018). Decreased levels of G9a and H3K9me2 have been associated with low anxiety

phenotypes (Anderson et al., 2018). It is possible that this decrease in G9a/GLP and subsequent decrease in H3K9me2, permits *Bdnf* expression that may account for increased *Bdnf* expression trends observed in reduced anxiety phenotypes. It is vital that one should not assume that an increase in *Bdnf* expression will decrease anxiety phenotypes, as depending on the age of the organism and the brain region undergoing BDNF-facilitated neurogenesis, BDNF may promote positive behavior learning such as fear extinction later in life, or negative memory-associated learning, such as fear-conditioning at younger ages.

Other implicated genes in anxiety studies include *Fkbp5* (Hartmann et al., 2015; St-Cyr et al. 2017; Schmidt et al., 2019) and the histone modifying enzymes - HDAC5 (Renthal et al., 2007), HDAC8 and the HMT Suv39h1 (Suri et al., 2013). Again, it is of great importance to understand that other epigenetic factors underlie AD pathology via crosstalk (Schiele et al., 2020). ATP-dependent chromatin remodeling factors, such as SNF2H and CHD3, have been shown to be differentially expressed in the amygdala and ventral hippocampus respectively, of high anxiety mice (Wille et al., 2016). These chromatin remodeling factors, such as CHD3, are able to form NuRD complexes – large protein remodeling complexes, with HDAC1/2 (Wille et al., 2016). This mechanism highlights the notion that no single epigenetic marker works independently, rather they work in concert with other chromatin remodeling factors in order to alter chromatin states and modulate gene expression (Wille et al., 2016).

Limitations & Future Directions

Though there is a substantial amount of literature investigating specific DNA methylation and histone modification trends underlying the anxiety phenotype, consensus is rarely unanimous or straightforward due to several factors:

1. While studies conducted in humans tend to utilize blood and saliva samples - peripheral fluids do not accurately represent brain neurochemistry involved in AD as each tissue type carries its own unique pattern of epigenetics (Braun et al., 2019). However, longitudinal studies in neonatal participants of cord blood methylation could elucidate potential markers for risk or resilience towards developing an AD or other psychiatric conditions in later life. Additionally, post-mortem studies utilizing human brain samples of known AD patients would add diversity to the epigenetic biomarkers currently reported. Likewise, the development of investigative tools like BECon, that aim to interpret blood-based DNA methylation findings within the context of the brain, could encourage more human-focused psychiatric studies in the future (Edgar et al., 2017).
2. Though there are multiple methods for modeling anxiety in animals such as rodents, as well as testing and measuring anxiety-like behaviors in these models, directly relating findings to human psychopathology often runs the risk of oversimplification as psychiatric disorders are characteristic of human beings and our complex thought processes and emotionality. Use of primate models, such as the macaques in Alisch et al.'s 2014 study, would be ideal as they are more closely-related to humans.
3. Majority of anxiety studies tend to focus on either specific brain regions, omitting other relevant structures, or, pool all samples by using the whole brain. Each brain region carries its own pattern of epigenetic alterations depending on the nature of the stressor and timing of application. Focusing on a single brain region within a study falls short of constructing a bigger picture of brain pathways altered in

anxiety, whilst global measurements are often biased, crediting overall epigenetic changes to the anxious phenotype without regard for local changes. Studies that aim to investigate multiple brain regions individually within a single model, could help to construct a larger picture of the anxiety phenotype on an epigenetic level.

4. Likewise, while anxiety-based studies tend to focus on specific DNA methylation marks or histone modifications, it is important to not lose sight of the fact that these regulatory markers work in tandem to alter gene expression, and are not independent of each other. Oftentimes, signaling cascades involving these modifications are not investigated. While future studies could focus on newly studied epigenetic modifications like histone serotonylation and dopaminylation, or lesser investigated mechanisms within the field of anxiety such as nucleosome positioning and microRNA regulation of protein-coding genes, more holistic studies that attempt to tie DNA methylation patterns and DNMT expression to histone modifying enzyme expression and histone markers could lead to a better overview of the bigger picture underlying the anxiety phenotype.
5. Lastly, many studies conclude before elucidating target genes, specific methylation sites and gene expression patterns under the regulation of these differential epigenetic modifications. It would be prudent for future studies to fill in the gaps of previous work.

Conclusion

The development of an anxiety disorder, like other psychiatric diagnoses, is multifactorial, with both an individual's genes and their environment contributing to disease pathology. This is reflected in incidences of comorbidity, such that individuals fitting the criteria

for an AD diagnosis, may also be diagnosed with a substance abuse disorder or a mood disorder, such as depression. This reality, coupled with the notion that each biological component underscoring the anxiety phenotype is complexly layered, means parsing out individual causes of anxiety-specific psychopathology remains a challenge.

This area of neuroepigenetic research aims to identify patterns of epigenetic modifications that have an overall impact on genes and corresponding protein products that play key roles in sustaining ADs. Thus, the research reviewed here not only strives to better understand the ongoing pathology of ADs by identifying corresponding patterns across studies, but to also provide insight on how to efficiently address these underlying aberrations in order to alleviate symptom severity and improve quality of life. Notably, rodent models have been a great resource for discerning the efficacy of anxiolytic and antidepressant medications on psychopathology-related behavior, and though differential epigenetic modifications vary between model types, these animals can be used to further elucidate the mechanisms through which these drugs act, for safer and better targeted treatment regimens in the future.

While the lack of consensus amongst DNMT levels across anxiety models make these enzymes a harder treatment target group, possible anxiety treatments include more specific HDAC inhibitors, since this class of drugs have been shown to encourage *BDNF* expression and neurogenesis, a phenomenon that may bolster CBT and fear memory extinction when treating ADs. The detection of key proteins such as FKBP5, which when downregulated showed rescue from the anxiety phenotype, also offer attractive avenues for future pharmacological interventions. Additionally, development of programs or algorithms that can bridge the gap between data obtained from peripheral tissue samples as biomarkers for the information gathered

from the CNS, would be beneficial to the entire field of psychiatry as a diagnostic tool that can be used to screen for populations at risk.

The field is moving in a positive direction, but it is imperative to continue with additional collaboration between laboratories and institutes, harmonization of human and animal studies, and bridging findings from past research.

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Table Legends

Table 1. Summary of stress models utilized to create anxiety models in rodents.

Table 2. Summary of assays used to measure the anxiety phenotype in rodent models.

Table 3. Summary of gene abbreviations.

Table 4. Summary of DNA methylation patterns and differential gene expression levels. Genes are italicized. (Me) denotes methylation reported. HADS-A: Hospital Anxiety & Depression Scale.

Table 5. Summary of differential histone modifications marks and histone modifying enzymes in stress/anxiety models.

Figure Legends

Figure 1: Multilevel model of risk and resilience in anxiety disorders. Genetic factors interact with environmental stressors and coping-related protective factors that determine risk or resilience via epigenetic mechanisms that regulate neural and neuroendocrine networks in the CNS, as well as the hormonal pathways of the HPA Axis. Source: Schiele& Domschke, 2018.

Figure 2: Schematic depiction of possible histone modifications marks on each histone (H2A, H2B, H3 & H4). Yellow triangle – acetylation; green square – permissive methylation; red square – repressive methylation. Note that multiple marks can occur at the same residue ex. lysine (K) 9 on histone 3 (H3). Adapted from Sun et al., 2013.

Figure 3: Visual representation of the HPA Axis and the Stress Response. Source: Edwards & Guilliams, 2010.

Figure 4: Tests used to assess anxiety in rodent models. (A) Light-Dark Box apparatus. The light compartment is twice the size of the dark compartment. Source: Riebe & Wotjak, 2012. (B) Elevated-plus maze apparatus with open and closed arms on a movable platform. Source: Walf & Frye, 2007. (C) Image of a video recording of the open-field test. Zones – blue: peripheral zone, green: inner zone, yellow: center zone. Note that not all open field tests are subdivided as shown here. Source: Barker et al., 2017.

Figure 5: Bisulfite conversion steps of DNA. Boxes indicate unconverted 5mC throughout the process. Adapted from Li & Tollefsbol, 2011.

Figure 6: Immunohistochemical staining of mutant *FKBP51*^{F67V} in the BLA of mice that exhibited anxiety-like phenotypes. Source: Hartmann et al., 2015.

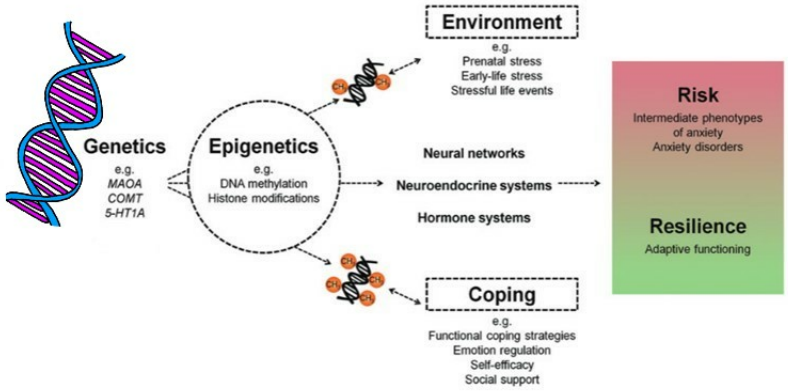
Figure 7: DNMT1 binding to GAD67 in PRS mice. (A) Graph showing DNA methyltransferase 1 (DNMT1) binding differences to specific promoter regions of GAD67 between control and PRS groups. (B) Graph showing MeCP2 binding differences to specific promoter regions of GAD67 between control and PRS groups. Source: Zhu et al., 2018.

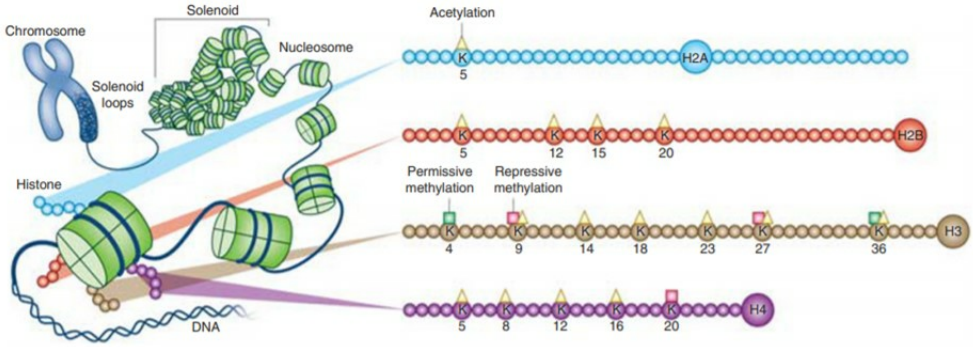
Figure 8: Differential DNMT expression levels in (a-c) E19 embryos and (d-f) P90 adult rats exposed to prenatal GIH. *denotes significant differences. The decreased expression of Dnmt3b persists from the embryonic stage in GIH adults. Source: Wang et al., 2013.

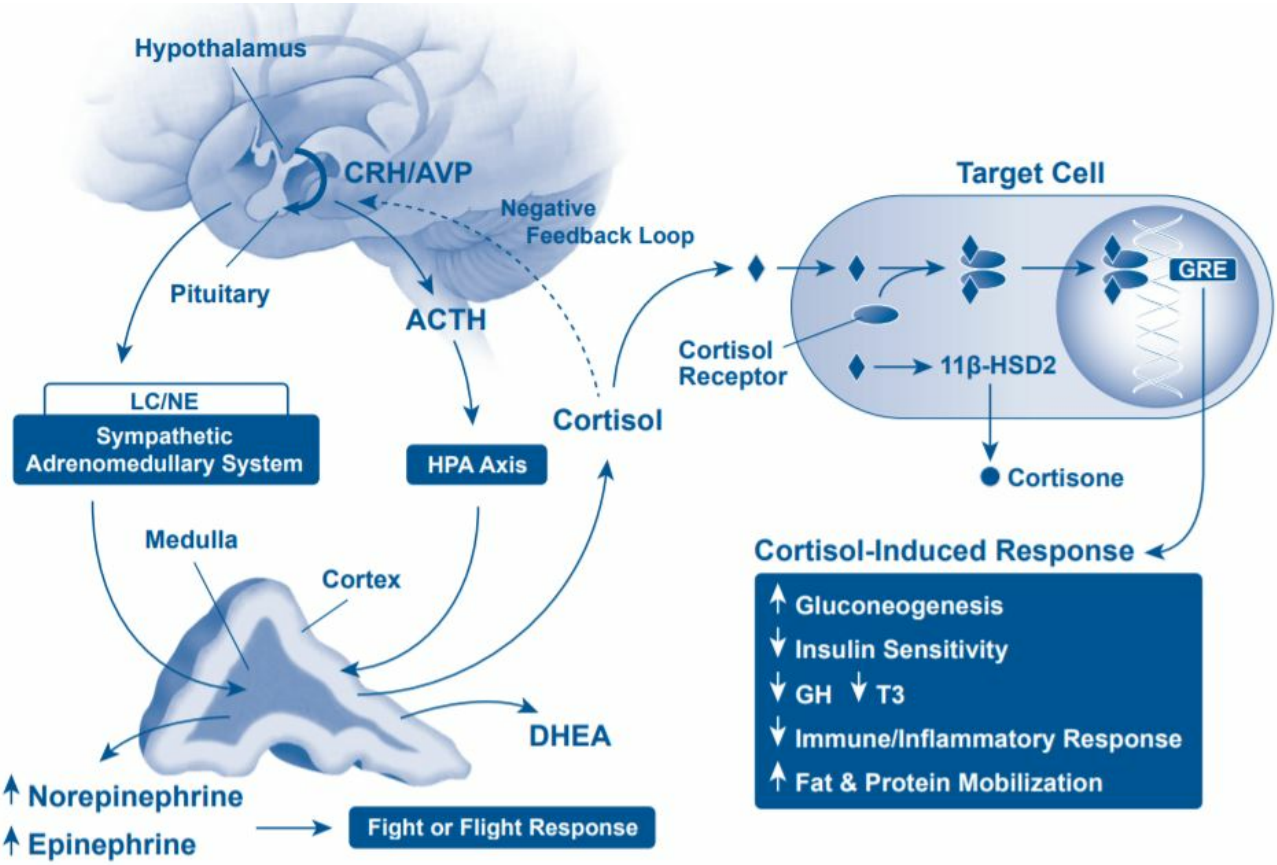
Figure 9: Schematic representation of the proposal regulation of BDNF expression by MeCP2. Source: Martinowich et al., 2003.

Figure 10: Differential H3K9me2 and BDNF IV expression. (B), (F) & (J) show the fold change in H3K9me2 between control and early stress animals (ages: P21, 2 months, 15 months); (C), (G) and (K) show differential BDNFIV expression levels in the hippocampal DG. Adapted from: Suri et al., 2013.

Figure 11: Hypothetical representation of the mechanistic role of HDAC inhibitors VPA and TSA. Source: Abel & Zukin, 2008.







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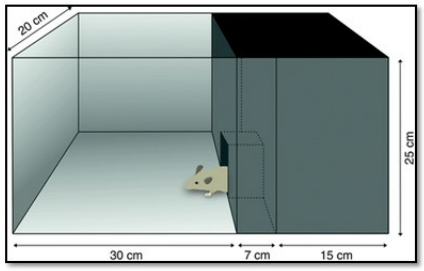

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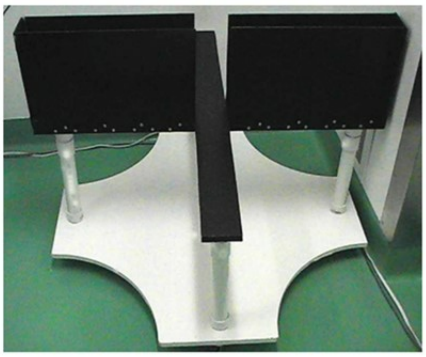

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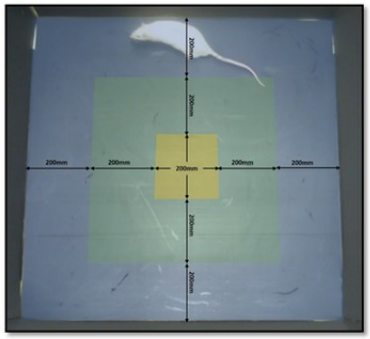

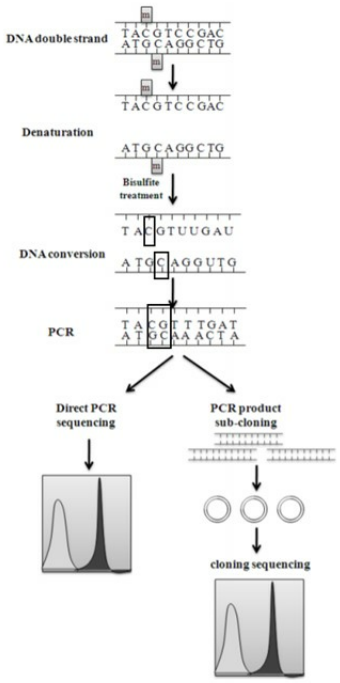

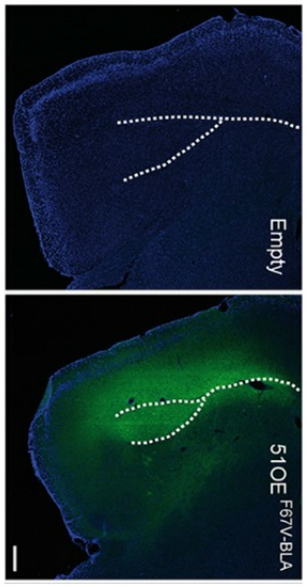

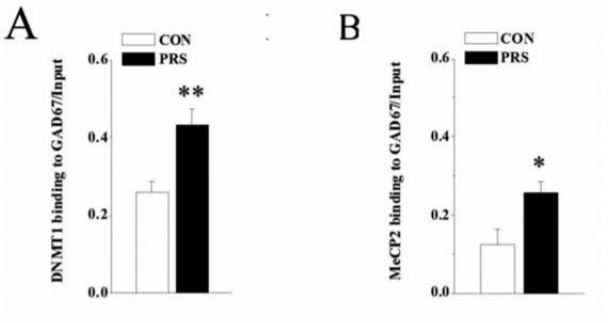

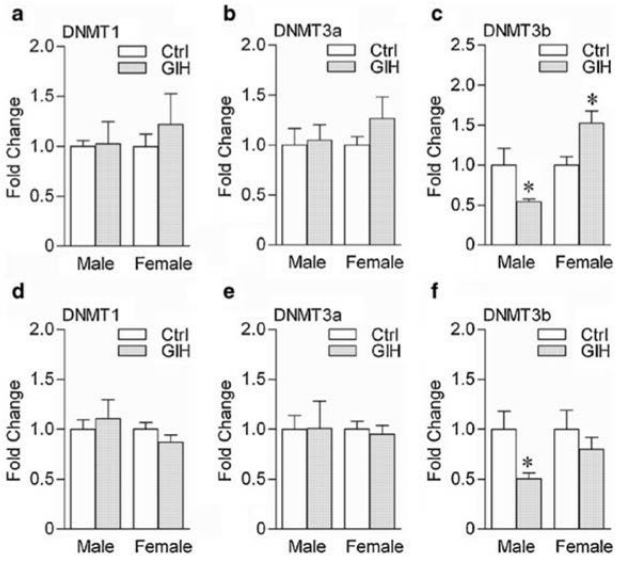

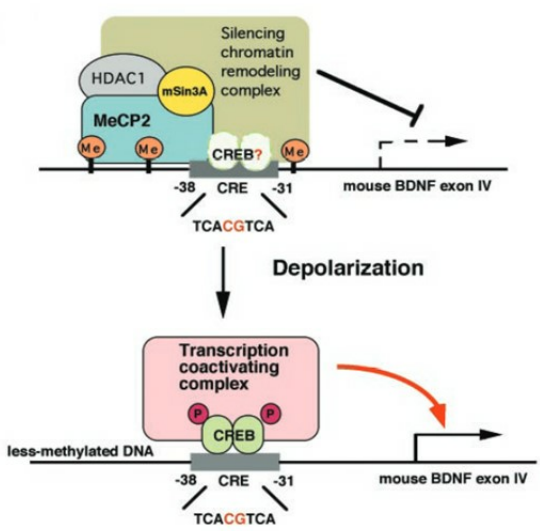

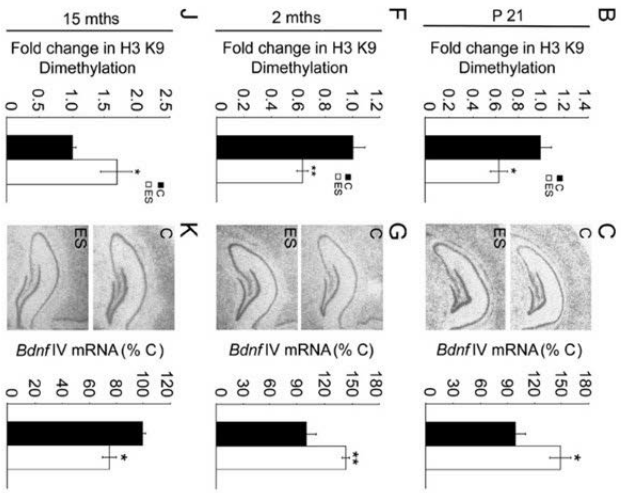

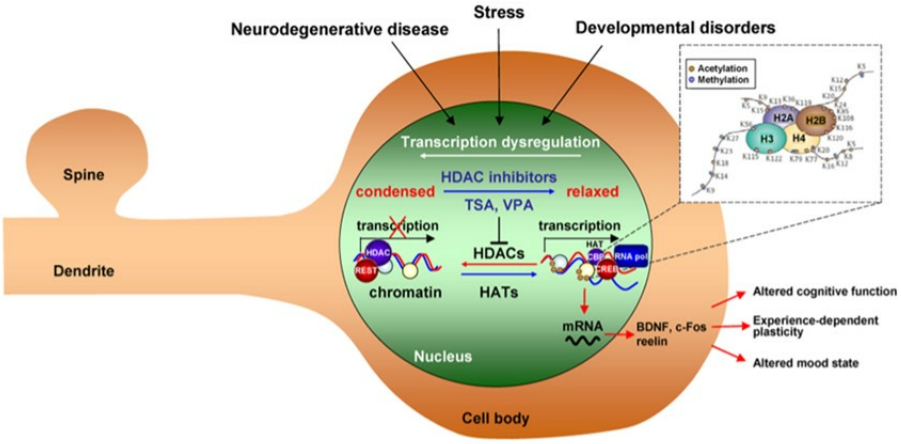

Stress Model	Description	Source
Chronic mild stress (CMS); chronic unpredictable stress (CUS); chronic variable stress (CVS)	- originally developed by Paul Willner as a model for depression, involves the subjugation of animals to a series of multiple, unpredictable stressors over a prolonged period - may vary in the combination and duration of stressors - many limitations, such as difficulty to replicate	Willner P., 1997; Lezak et al., 2017
Chronic social defeat stress (CSDS)	- initially developed to model depression by Avgustinovich et al. (2005); was popularly adapted to investigate anxiety phenotypes by Krishnan et al., (2006) - study animal is introduced to the cage of a larger, aggressive animal (often a different strain of rat or mouse) - study animal is consider the 'intruder'; aggressor animal is considered a 'resident' - exposure lasts for ten minutes for ten days, but may differ based on the researcher's goals - some animals may overcome CSDS-induced anxiety and are considered models for studying anxiety resilience - limitations: sex-related differences (females tend not to participate in territorial related aggression); and injury to the model animal by the aggressor animal	Avgustinovich et al., 2005; Krishnan et al., 2007; Lezak et al., 2017
Prenatal Stress	- originally utilized in rats by William Thompson (1962), this stress model involves the application of a stressor to a pregnant dam; later adapted to study anxiety-like behaviors in offspring by other researchers (Vallée at al., 1997) - considered a developmental form of stress - stressors include footshock, restraint stress, subjection to endocrine disrupting chemicals etc.; for more comprehensive reading on variations used in this model, please refer to *Prenatal stressors in rodents: Effects on behavior* (Weinstock, 2017)	Thompson et al., 1962; Vallée et al., 1997; Lezak et al., 2017
Postnatal Stress	- administration of a stressor following the birth of pups and was first reportedly used by Krzysztof Janus (1987) - considered a developmental form of early life stress - the most commonly employed stressor is maternal separation in rodents, the time of separation is crucial for inducing anxiety-like phenotypes: separation at postnatal day (PND) 3-4 tends to induce anxiety-like behaviors, whilst separation at PND 11-12 has been shown to cause the opposite effect of hyporesponsiveness	Murthy & Gould, 2018; Lezak et al., 2017; van Oers et al., 1998

Test	Characteristics	Source
Light-Dark Box Assay	• box apparatus is divided into two sections: the smaller dark 'protected' side (minimally lit with black walls), and the larger light 'unprotected' side (brightly illuminated with white walls) • relies on the principle of a rodent's innate aversion to light and exposure to predators as a prey animals • Shorter latency periods entering the light side, and/or longer periods spent here, are interpreted as reduced anxiety-like behaviors • measures the approach-avoidance behavior	Crawley & Goodwin, 1980 Campos et al., 2013 Lezak et al., 2017
Elevated Plus Maze (EPM) Test	• consists of two open, or unenclosed arms opposing two enclosed arms in the shape of a plus sign • apparatus is elevated several feet from the ground • Animals are placed at the center of the EPM and allowed to explore freely for five minutes • exposure created by open arms is associated with anxiety-like behavior, such as increased defecation and corticosteroid levels • more time spent in, as well as higher percentage of entries into the open arms of the EPM, are interpreted as reduced anxiety-like indices • measures approach-avoidance behaviors	Handley & Mithani, 1984 Korte, 2001 Campos et al., 2013 Lezak et al., 2017
Open Field Test	• consists of an open box divided into layers of rings from the center of the box to the corners • the time spent in the middle where hypothetically the animal is most exposed and vulnerable, is compared to the amount of time spent hugging the safer corners of the box • the number of times ventured towards the center of the box is also recorded • reduced anxiety is inferred if the animal tends to venture out from peripheral zones, or spend longer periods in the more central zones of the open box • relies on instinctual fear responses to predators	Lezak et al., 2017

Protein Product	Gene Denotation
Glutamate Receptor Subunits	*Grik1, Grik2, Grin1, Grin2b, Grin3a, Grm5, Nr2b*
GABA Receptor Subunit	*Gabra2, Gabbr1, Gabbr2*
Mineralocorticoid Receptor	*Nr3c2*
Glucocorticoid Receptor	*Nr3c1*
Jagged-1	*JAG1*
B-cell lymphoma/leukemia 11A	*Bcl11A*
Corticotropin Releasing Hormone/ Factor	*CRH/CRF*
Glutamic Acid Decarboxylase	*GAD67*
CRH Receptor	*Crhr1*
FK506 binding protein 5	*FKBP5*
Histone-lysine N-methyltransferase SUV39H1	*SUV39H1*
Euchromatic Histone-lysine N-methyltransferase	*EHMT2, G9a*
G9a-like Protein	*EHMT1, GLP*

Marker	Sample Retrieved	Anxiety Model	Reference
↕***Dnmt1***	Hippocampus (CA1, CA3) Amygdala (medial, basolateral & lateral nucleus)	bLR animals (rats: P7-P14)	Simmons et al., 2012
↓***Dnmt3a***	Peripheral blood	Anxious young adults (measured with HADS-A)	Murphy et al., 2014
	Medial Prefrontal Cortex (mPFC)	Adult CSDS (9 weeks) mice (sacrificed 4 wks after CSDS)	Elliot et al., 2016
↓***Dnmt3a*** ↓***Dnmt3b*** ↓***Grin2b*** ↓***Gabar2*** ↑***Grin1***	Hippocampus	Low-methyl diet adult mice (6 & 12 wks) (LOW ANXIETY)	Ishii et al., 2014
↑***NR3C1*****1F (Me)**	Peripheral whole blood	Adolescents (mean age = 16 yrs) who reported SLE's	van der Knaap et al., 2014
↓***NR3C1*****1F (Me)**	Leukocytic blood	Adults (18-65 yrs) who reported SLE's and diagnosed with variable ADs	Tryka et al., 2016
↑***NR3C1*** **(Me)** ↓**GRα**	Peripheral blood mononuclear cells (PBMCs)	Adults (mean age = 35 yrs) diagnosed with GAD	Wang et al., 2017
↓**GR** ↑***FKBP5***	Placenta	NR3C1$^{+/-}$ mice	Schmidt et al., 2019
↑***FKBP5***	Amygdala	Adult rats (25 wks) born to dams exposed to predator odors (prenatal/*in utero* stress)	St-Cyr et al. 2017
	Amygdala (basolateral)	Viral overexpression of FKBP51	Hartmann et al., 2015
↓***Dnmt1*** ↑***Gabra2*** ↓***GABA***	Whole brain	H67D male mutant mice (LOW ANXIETY)	Ye et al., 2018
↑***Dnmt1*** ↓***GAD67***	Amygdala (basolateral)	Female mice (P45) exposed to BPA in utero	Zhou et al., 2013
		Female mice (P60/P70) exposed prenatally/in utero to maternal restraint stress	Zhu et al., 2018
↑***Crf*** ↓***Dnmt3b***	Hypothalamus (PVN)	Adult CSDS mice	Elliot et al., 2010

↑*Crf* ↑Cortisol	Hypothalamus	PRS adolescent rats (P38) born to dams subjected to restraint stress	Xu et al., 2014
↑*Crf* ↑*CRHR1*, ↓*Dnmt3b*	Hypothalamus (PVN)	Male adult (P90) rats exposed *in utero* to gestational hypoxia	Wang et al., 2013
↓CRHR1 (Me)	Peripheral whole blood	Adults (mean age = 35 yrs) diagnosed with Panic Disorder	Schartner et al., 2017
↑*Crhr1*	Amygdala (basolateral)	HAB mice bred for a high anxiety trait, LAB mice exposed to CMS	Sotnikov et al., 2014
GRIN1, GRM5* ↓*BCL11A*, ↓*JAG1	Amygdala (central)	Anxious temperament rhesus macaques (mean age = 1.3 years)	Alisch et al., 2014

Marker	Sample Retrieved	Stress Model	Reference
↑H4ac ↑*BDNF* IV	Prefrontal Cortex	Footshock conditioned male mice (10-12 wks) with fear extinction learning (LOW ANXIETY)	Bredy et al., 2007
↓H3K9me2 ↑*BDNF* IV	Hippocampus	Male rats (P21 & 2mths) subjected to maternal separation	Suri et al., 2012
↑H3K9me2 ↓*BDNF* IV		Male rats (15mths) subjected to maternal separation	
↓H3K9me ↓H3K9me3	Hippocampus	Adult male rats (P70) subjected to CRS	Hunter et al., 2009
↓H3K9me ↓H3K9me3	Prefrontal Cortex	Young female rats subjected to early maternal separation (LOW FEAR-CONDITIONED STARTLE)	Kao et al., 2012
↑H3K9me3 ↓GR	Amygdala; Hippocampus	Adult bHR (LOW ANXIETY) rats	Chaudhury et al., 2014
↓G9a ↓H3K9me2	Whole brain	Adult mice treated with G9a inhibitors (LOW ANXIETY phenotype)	Wang et al., 2018
↑G9a ↑H3K9me2	Nucleus accumbens	Viral G9a overexpression in male adult rats (high anxiety phenotype)	Anderson et al., 2018
↓*HDAC2* ↑*HDAC3* ↓*HDAC8* ↓*Suv39h1*	Hippocampus	Young adult rats (2mths) subjected to early maternal separation (Anxiety phenotype)	Suri et al., 2013
↓H3K9ac ↓GR ↑*CRF* ↑SIRT6	Amygdala (central)	Male adult mice were infused with CORT (chronic anxiety)	Tran et al., 2014
↓H3K9ac ↓H4K12ac	Hippocampus	Adult male rats subjected to CVS	Ferland & Schrader, 2011
↓HDAC5	Nucleus accumbens	Mice subjected to CSDS (chronic anxiety)	Renthal et al., 2007
↑HDAC2 ↓H3K9ac	Amygdala (central)	Adult P rats bred for alcohol preference (anxiety phenotype)	Moonat et al., 2013