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Genome-wide studies of transcriptional dynamics in fear learning

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Abstract

Genome-wide studies of transcriptional dynamics in fear learning
By Sumeet Sharma

The consolidation of fear-related memories requires synaptic plasticity dynamics in many brain regions, with the basolateral amygdala (BLA) thought to be the site where cue associations with fearful physiological outputs are made. Herein we employed mRNA-sequencing and 5-hydroxymethylcytosine profiling to obtain a genome-wide view of transcriptional and epigenetic dynamics in the BLA during fear memory consolidation. We find that differentially hydroxymethylated regions (DHMRs) reside primarily within introns, exons, enhancer elements, mitochondrial DNA, and repetitive DNA. We find 727 genes to be dynamically expressed (DE), of which 108 also contain differentially hydroxymethylated regions (DHMRs). These DE genes encompass pathways in mitochondrial function, gene transcription, protein translation, and heat shock response. Within the DHMR-containing DE genes, we replicate *Fkbp5* dynamics in fear conditioning. *Fkbp5* is a well-known gene previously implicated in stress responses, fear processing, and fear- and stress-related disorders in humans. We find that *fkbp5* is modulated at both the transcriptional and DNA methylation level, and we identify CHH-context cytosines within intron 1 that are casual drivers of *Fkbp5* gene regulation. Finally, to better examine the dynamics of transcriptional dynamics in fear processing, we demonstrate the development of a robust novel behavioral procedure that results in stress enhanced fear learning and stress-dose dependent alterations in anxiety.

Genome-wide studies of transcriptional dynamics in fear learning

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Table of Contents

Table of Figures

Introduction and Historical Background

The goal of translational biomedical research is to advance our understanding of biology directly related to disease processes, in order to better treat those afflicted. In medicine in general, unbiased approaches to understanding disease pathophysiology have become increasingly powerful as DNA sequencing technologies have come to fruition. In the field of neuroscience and psychiatric disease in particular, the complexity of the central nervous system have made these unbiased approaches one of the best windows into the molecular underpinnings of disease. Genome-wide association studies (GWAS) and gene-by-environment (GxE) investigations have identified a variety of fruitful associations with disease that have pointed us towards molecular and circuit-level targets that may shed light on novel approaches to diagnose and treat mental illness. Similarly, animal models of cognition have also begun to use unbiased methods to understand biology. At the level of neural circuitry, unbiased molecular profiling approaches (epigenetic, transcription, and translation of genes) are allowing the identification of relevant substrates of neural function and dysfunction. Broadly, the aim of this thesis is to implement unbiased methods to elucidate the molecular pathways regulated by fear, and to probe the relationships between key functional nodes, gene function, and behavior. Towards the goal of translation, we aimed to take two complementary approaches towards the prioritization of gene pathways associated with fear learning in the amygdala.

Initial, unbiased genomic profiling in the mouse was used to identify differentially regulated gene pathways as well as genes with dynamic 5-hydroxymethylcytosine (5hmC). Then findings from genetic association studies were used to prioritize the genes that were replicated in independent validations of the genome-wide results. This heuristic allowed us to focus our

manipulations on gene pathways that are differentially regulated by fear learning processes in rodents, and are potential etiological agents in patients with PTSD and other stress- and anxiety-related disorders.

Genetic Epidemiology

We begin first with a discussion of how genetic susceptibility to PTSD was established. Much of this discussion has been adapted from two reviews I have produced during my thesis training. (Sharma, Powers, Bradley, & Ressler, 2016; Sharma & Ressler, 2019) The first step in studying disease genetics is determining the heritability of a particular disorder, which has historically been carried out through epidemiological studies. One of the earliest studies of psychiatric heritability was conducted in 1911, when Canon and Rosanoff used family pedigrees to search for patterns of Mendelian inheritance in psychiatric patients. (Zhang, 2011) This was a precursor to large-scale genetic epidemiology studies (e.g., twin-, family-, adoption-, and other population-based studies) that have provided a necessary first step in establishing heritability and exploring genetic interactions in stress and anxiety disorders.

The two necessary factors for the development of PTSD, also known as the stress-diathesis hypothesis, are: 1) undergoing a traumatic experience and 2) having an underlying susceptibility to disease. One could imagine that susceptibility to be driven by genetic predispositions, environmental influences on developmental processes (e.g. early life stress, or stress experienced *in utero*) or the combination of both. Disentangling these interactions at the level of specific polymorphisms via genetic association studies is in its infancy, as we will discuss later on. However, epidemiological approaches can be very informative as to the

magnitude of the role genetics play in heritability and to which environmental associations we should pay attention.

Twin studies were the first approach used to define genetic heritability of PTSD, by comparing the incidence of PTSD in pairs of monozygotic and dizygotic twins. Studies found a 46% heritability in a combined sample of male and female twins, and 72% in an all-female sample of twins. (C E Sartor et al., 2011; Carolyn E Sartor et al., 2012) Twin studies have also demonstrated that genetic variation contributes to the risk of trauma exposure. A 2002 study suggested that risk for experiencing assaultive traumas (such as sexual assault or robbery) were moderately heritable, while non-assaultive traumas (such as natural disasters and car accidents) did not have a detectable genetic component. (Stein, Jang, Taylor, Vernon, & Livesley, 2002) While initially controversial, with the concern of 'blaming the victim', at the level of epidemiological studies, such findings suggest that factors such as risk-seeking behavior or inattention to danger could help to explain heritability of trauma exposure. Together these data suggest that there is genetic susceptibility both to experiencing particular social trauma and to developing PTSD in the aftermath of trauma exposure.

The incidence of PTSD is directly related to the type, severity, and pervasiveness of trauma in a population. People who have experienced more trauma are at higher risk for the eventual development of PTSD. For this reason, GWAS approaches have taken the approach of using trauma-matched controls – so that the associated variants will better delineate the risk alleles that characterize the 5-15% of the population susceptible to PTSD. Longitudinal epidemiological studies of military personnel have further characterized risk versus resilience.

Studies in military personnel who had directly experienced combat in the Vietnam War demonstrated a 19% lifetime risk for PTSD, 10-year post-war rates as high as 28%, and as many

as 11% of veterans continued to demonstrate PTSD symptoms up to 40 years after combat. (Dohrenwend et al., 2006; Marmar et al., 2015) In studies of soldiers who have served in Iraq or Afghanistan, the incidence of PTSD is proportional to the severity of the trauma experienced. However, a ceiling is observed in that the maximal incidence appears to be about 25-30% in such studies, suggesting a threshold after which the dose-response effect of trauma-severity on population risk does not continue to increase. Another result from these longitudinal studies has been the characterization of the diversity of outcomes post-trauma, including spontaneous recovery (recovery from disease without any treatment) and delayed PTSD (the development of more severe symptoms sometime after the initial traumatic experience). (Smid, Mooren, Van Der Mast, Gersons, & Kleber, 2009)

Another important observation from epidemiological studies is that the lifetime risk of PTSD in women is double that of men. (Norris, Foster, & Weisshaar, 2002) An active area of inquiry is to understand whether this enhanced risk stems from increased exposure to traumatic events (i.e., sexual abuse and rape), or whether there may be a differential sex-dependent genetic predisposition to PTSD. The data are mixed, and it remains possible that both a higher exposure to particular traumatic events and genetic predisposition may both play a role. The majority of studies suggest that even when differences in trauma experience are accounted for, sex differences in PTSD incidence persist. The most convincing evidence comes from studies of matched trauma histories, wherein the greater female risk for PTSD cannot be accounted for by greater exposure to trauma, and this finding appears to be stable across a variety of types of trauma. (N Breslau, Chilcoat, Kessler, Peterson, & Lucia, 1999; Naomi Breslau & Anthony, 2007) However, conflicting evidence was reported in a 2006 study of intimate partner violence, suggesting that once differences in trauma exposure are accounted for, the increased risk for

PTSD in females disappears. (Cortina & Kubiak, 2006) Indeed, the authors of this study make valid criticisms of the questionnaires that are used to quantify types of trauma and suggest that improvements could be made to gather data that better reflects the reality of the female trauma experience. More work is required to generate the large, representative datasets needed to answer these questions. (Yehuda et al., 2015)

Finally, one of the most powerful influencers of multiple future pathologies, including PTSD, is early life stress. (Bremner, Southwick, Johnson, Yehuda, & Charney, 1993; C Heim & Nemeroff, 2001; Christine Heim & Nemeroff, 2002; Kaufman, Plotsky, Nemeroff, & Charney, 2000; Lang et al., 2006; Stovall-McClough & Cloitre, 2006) A plethora of epidemiological evidence has established the role of early life pathology in PTSD risk, and preliminary evidence has implicated several genetic variants in mediating the deleterious effects of early life stress on future psychopathology, including *FKBP5*, which will be discussed further below.

The first step in unraveling which genes play important roles in disease is to understand the gene variants which contribute to a main effect on PTSD risk, independent of environmental variables. In the past 10 years, several candidate gene associations have elucidated sex-specific and gene-by-environment (GxE) interactions with trauma. More recently consortium efforts have gained momentum to aggregate the vast number of samples needed for GWAS to identify strongly associated variants.

Genetic Association Studies

The purpose of GWAS is to identify loci in the genome where genetic variation is associated with the presence of disease. These disease-associated variants are thought to increase the risk of developing the related disorder, but mechanistic studies are required to confirm the

influence of a genetic variant on disease pathophysiology. (Hirschhorn, Lohmueller, Byrne, & Hirschhorn, 2002) In contrast to G×E studies, GWAS query the main effect of a genetic variant. The statistical definition of a main effect is the effect of an independent variable on a dependent variable, averaging across all other independent variables involved. In GWAS terms, that is equivalent to determining the association of a particular genetic variant with a disease and averaging across all other measured environmental variables. G×E studies are an extension of GWAS, wherein G×E studies also consider the environment as a variable. In a G×E framework, the environment can be considered the pathogenic or etiologic factor, and the genetic variant is contributing to the susceptibility to that environmental pathogen. (Kim-Cohen et al., 2006) In the case of PTSD, exposure to the traumatic experience is the equivalent of the environmental pathogen or insult.

A genetic variant is any portion of an individual's DNA sequence that differs from the reference human genome sequence. The majority of genetic association studies focus on single nucleotide polymorphisms (SNPs) as the source of genetic variation, in large part because of the rapid technological advancements and cost-effectiveness of SNP genotype arrays. However, chromosomal rearrangements (duplications, deletions, inversions, and translocations) can also be quite common, and SNP-based GWAS can be extended to query copy number variation. (McCarroll, 2008; Mills et al., 2011) Evolutionary models of complex diseases posit that both common variation and rare variation in the genome contribute to disease. (Cichon et al., 2009) A common SNP is defined to have a minor allele frequency (MAF) of at least 5%, whereas a rare variant is defined by a MAF of 1% or less; at the extreme, an extremely rare variant may only be present in a single individual. The MAF is defined as the frequency of the least common allele in a population.

The common disease-common variant hypothesis posits that some portion of disease heritability must lay in common variants, and it assumes that testing SNPs in enough cases and controls can collectively identify common SNPs with small individual effects on disease status. It is more challenging to draw statistically significant conclusions about rare variants, as their prevalence is very low; however, the 1000 Genomes Project and other large-scale efforts have allowed us to query SNPs with a MAF in the population as low as 0.01%. (Abecasis et al., 2012; Solovieff et al., 2014) The rich catalog of human variation that has been produced by the HapMap Project and the 1000 Genomes Project has greatly contributed to the advancement of genetic association studies, just as consortium efforts have advanced the collection of population-level genetic variation.

The need for greater statistical power in psychiatric genetics led to the formation of the Psychiatrics Genomics Consortium (PGC). As in other disciplines of biomedical research, it quickly became apparent that many variants identified through GWAS are of very small effect size, i.e., their contribution to disease is small. Thus, discovering these variants of small effect requires very large samples. Logue et al. (2015) calculated that tens of thousands of subjects will be required to discover disease-associated SNPs with MAFs of 5-20% in the population. (Logue et al., 2015) The PGC has facilitated collaborative efforts in studies of genetic association for schizophrenia, bipolar disorder, and major depressive disorder (MDD), among others. Schizophrenia represents the major GWAS success in psychiatry so far. To date, the latest GWAS meta-analysis has revealed over 108 loci as being genome-wide significant. (Consortium, 2014) An association at a genome-wide significance level means that a genetic variant is associated with cases over controls, with $p < 5 \times 10^{-8}$, based on a conservative multiple test correction of $p = 0.05$ divided by 1 million SNP tests. This p-value is based on statistical

estimates assuming that all common SNPs have been tested. Although other non-frequentist statistical measures (Bayesian approaches) have been used that also have merit, the majority of studies to date utilize significance testing with *p*-values as the measure of statistical significance. (Sham & Purcell, 2014)

PTSD GWAS

In the sudy of PTSD genetic association, to date the published GWAS results have largely been underpowered to detect statistically significant loci that have replicated within and across studies, though some have yielded genome-wide significant loci ($p < 5 \times 10^{-8}$) in the discovery cohort. See Nievergelt et al. for a review of the gene variants that have reached genome-wide significance in any study. (Nievergelt et al., 2018) The largest published PTSD GWAS to-date is the Freeze 1 dataset of the PGC-PTSD, which comprised 11 multiethnic cohorts with 5000 cases versus 15000 mostly trauma-exposed controls (87.7% trauma-exposed). This analysis was not sufficiently powered to identify PTSD associated SNPs at a genome-wide significant p-value. (L. E. Duncan et al., 2018) The next iteration of the psychiatric genomics consortium's PTSD GWAS dataset, freeze 2, will include 32,000 cases and 100,000 trauma-exposed controls. (Nievergelt et al., 2018) This sample size is approaching the level at which genome-wide significant loci have been discovered for other psychiatric disorders, such as schizophrenia.

A number of analyses were carried out in the latest PGC-PTSD GWAS that elucidated some interesting aspects of the underlying genetic architecture of disease, including heritability and cross-disorder genetic overlap. The authors estimated the overall molecular heritability of PTSD to be ~15%, however, they found much higher estimates for females compared to males. Female heritability was calculated to be 29% whereas heritability for males was not significantly greater

than 0% - which may reflect a lower genetic component to disease, but is likely inaccurate due to relatively the small sample size and the heterogeneous population available.

In contrast to twin studies, these early SNP-based heritability estimates for PTSD are much lower. Previous work from twin studies has estimated heritability for PTSD in the ranges of 13-34%, 46%, and 72%. (Cantor, Lange, & Sinsheimer, 2010; C E Sartor et al., 2011; Carolyn E Sartor et al., 2012; True et al., 1993) However, the underpowered sample size of this latest PGC-PTSD study resulted in a low heritability z-score of 3.0 – a score influenced by the sample size, SNP-based heritability, and the proportion of causal variants. (Hill et al., 2016) Thus, the heritability estimates are more speculative than they will be in a larger dataset. Notably, investigations of heritability estimates in other traits, specifically schizophrenia and height, have suggested that gene-based heritability can be more fully accounted for if all SNPs queried, not just those that meet stringent p-value cutoffs, are included in the analysis, but this requires larger sample sizes. (Loh et al., 2015; Yang et al., 2010) Importantly, SNP-based heritability only including common variant SNPs is never expected to be as high as twin study estimates, since those 'true' heritability studies include common variants, but also all rare variants, insertions/deletions, gene x environment interactions, and even epigenetic inheritance – in short, all forms of heritability.

Analysis of other well-powered datasets suggests not only that genetics can indeed account for a large fraction of heritability, but also that most complex traits are extremely polygenic. Recent advances in analytical approaches of GWAS data suggest that the inflation of GWAS test statistics in well-powered datasets may be the result of polygenicity rather than genomic inflation. (Bulik-Sullivan et al., 2015) The next iteration of the PGC-PTSD dataset will likely provide a clearer picture of the molecular heritability of PTSD.

To analyze cross-disorder genetic correlation, the PGC-PTSD authors were similarly limited by the size of the dataset, and so limited their comparisons to schizophrenia, major depressive disorder, and bipolar disorder. Notably, using different polygenic risk score approaches, significant overlap was observed between PTSD and all three other psychiatric disorders, consistent with the shared heterogeneity of risk across disorders. (L. E. Duncan et al., 2018)

PTSD GxE Studies

The study of G×E interactions speaks to a question at the core of mental health and the study of human disease in general: To what extent do individual genetic variation and environmental context interact to influence the etiology of disease? The conception of G×E represents the realization that for many disorders, the effect of an external stimulus, be it an infectious agent, toxin, or physical or psychological trauma, depends on the unique genetic makeup of each individual. In the realm of stress and anxiety disorders, genetic variation may predispose individuals to resilience or susceptibility to environmental stressors, which may then result in the development of psychiatric disorders. This also means that without exposure to those environmental stressors, the negative outcome may not occur; thus, it is the interaction between genes and environment that is critical for the expression of the phenotype of interest.

G×E interactions represent our understanding of the shared influence that genes and the environment play in the development of mental disorders. Statistically, an interaction between two variables means that the outcome (disease) depends on both variables. For example, without knowing the genetic variants present in an individual, it is impossible to know the relative risk for development of PTSD in the aftermath of a traumatic event; vice versa, without knowing what traumatic experiences the individual has encountered, it is impossible to know whether he

or she will develop PTSD based on genetics alone. Thus, both components must be known to evaluate the etiology of disease.

In the field of PTSD genetics, the contingency of PTSD on exposure to trauma makes it particularly salient to include environmental measures of trauma into statistical models of genetic association. However, there are many statistical challenges to this approach, many of which have not been sufficiently accounted for in analyses to date. Covered extensively elsewhere, specific difficulties include properly scaling measurement variables, controlling for the effects of covariates on interaction, and a deeper understanding of potential nonlinear relationships between predictors and the outcome of interest that may masquerade as true associations. (Border & Keller, 2017; Keller, 2014; Moore & Thoemmes, 2016) Furthermore, it is now being appreciated that the candidate gene variants that have historically been employed in genetic association studies are not likely to be significant drivers of disease in human populations. (Farrell et al., 2015; Johnson et al., 2017)

In principle, it may be possible that certain genetic associations will not be detectable without including an environmental component in the statistical model. A simple example would be a type of cross-over interaction, wherein alleles that increase the risk for a certain disorder in a particular environment may confer protection to that same disorder in another environment. (Sharma et al., 2015) An example would be if a polymorphism in a gene interacts with early life stress, such that in the context of early life stress, one allele confers protection to future psychopathology while the other confers risk; however, in the context of a “less” stressful early life experience, those relationships are reversed. Some in the field argue this type of interaction is unlikely, however, more investigation is required to rule this possibility out. (Laramie E. Duncan, Pollastri, & Smoller, 2014) Furthermore, given the small effects any particular genetic

variant exerts on disease risk, as has been observed for schizophrenia and other psychiatric disorders with well powered GWAS, it is likely that much larger sample sizes and a more sophisticated statistical approach will be required to properly answer this question.

However contentious GxE studies have been, the contribution of environmental variables to psychopathology is clear and unbiased approaches to identify molecular correlates of environmental influences are needed. It is possible, and may be likely, that the large GWAS studies in PTSD are still insufficiently powered to capture polymorphisms that have been identified as disease associated in previous investigations, especially given that they may need to be studied in a gene x environment context. In the case of *FKBP51*, study-after-study has revealed widespread associations with psychopathology, stress responses, and outcomes such as drug-response in depression. While there is still contention in the field as to the significance of this gene to human disease, its predictive capacity in a variety of contexts and identification in multiple unbiased screens across human and animal studies , particularly fear learning in the mouse amygdala, points towards a key role for this gene in modulating disease-relevant biological substrates.

FKBP5

In 2008, an interaction between SNPs in **FK506 Binding Protein 51 (FKBP5)** and early childhood trauma (FKBP5 × childhood trauma) was found to influence the severity of adult PTSD symptoms in a population of urban, low-socioeconomic status African American. (Binder et al., 2008) A subsequent study replicated this finding in a larger cohort of subjects of African descent. (Xie et al., 2010) A replication study investigating G×E interactions in chronic pain patients in Pennsylvania demonstrated that the interaction between *Fkbp5* genotype and total

trauma exposure is associated with PTSD. (Boscarino, Erlich, Hoffman, & Zhang, 2012) No main effect for *Fkbp5* genotype and PTSD was detected in any of the preceding studies. Interestingly, adult trauma did not interact with *Fkbp5* genotype to influence PTSD symptoms, whereas follow-up studies have consistently reported the interaction of childhood trauma and *Fkbp5* variants to be significant. This suggests a developmental window in which environmental risk creates long-lasting molecular alterations in the FKBP5 pathway, which influence the development of PTSD in adulthood. The *Fkbp5* × childhood trauma interaction has also been shown to influence a variety of other psychiatric disorders and traits including depression, schizophrenia, aggression, psychosis, and suicide attempts. (Appel et al., 2011; Collip et al., 2013; Dackis, Rogosch, Oshri, & Cicchetti, 2012; Roy, Gorodetsky, Yuan, Goldman, & Enoch, 2010; Zimmermann et al., 2011) These diverse associations can be understood by the fundamental molecular role FKBP5 plays in regulating glucocorticoid signaling in the cell. FKBP5 exerts an inhibitory effect on *glucocorticoid receptor* (*GR*)-mediated signaling, acting in an ultrashort feedback loop of the hypothalamic–pituitary–adrenal axis (HPA axis).

FKBP5 is a cochaperone that associates with the heat shock protein 90 (HSP90) receptor complex. While its precise molecular assembly with HSP90 and steroid hormone receptors is incompletely understood, functional studies have demonstrated that FKBP5 negatively regulates steroid hormone receptor activity quite broadly – encompassing the progesterone, androgen, estrogen, mineralocorticoid, and glucocorticoid receptors. Downregulation of these hormone receptors is likely mediated by combinatorial reduced affinity of the receptors for their ligands mediated by the FKBP5 paralogue and oppositional regulator FKBP52 (also known as FKBP4), reduced trafficking of the receptors to the nucleus, reduced association with nuclear pore

complexes, and increased propensity to proteasomal degradation. (Echeverria et al., 2009; Galigniana et al., 2004; Storer, Dickey, Galigniana, Rein, & Cox, 2011; Wochnik et al., 2005)

FKBP5 has also been demonstrated to regulate the AKT serine/threonine phosphorylation pathway, specifically by recruiting the PHLPP phosphatase, which dephosphorylates AKT. Furthermore, in a cell culture model of cancer, reduced FKBP5 expression resulted in AKT hyperphosphorylation and reduced cell death following DNA damage. (Pei et al., 2009) This finding suggests that FKBP5 may influence other second messenger pathways in addition to hormone signaling.

Not only does FKBP5 regulate hormone receptor signaling, it is itself regulated by hormone receptor signaling, being upregulated by progesterone, androgen, and glucocorticoid receptor activation. (Hubler et al., 2003; Hubler & Scammell, 2004; Magee, Chang, Stormo, & Milbrandt, 2006) Thus, it is proposed that FKBP5 acts as an ultra-short negative feedback loop intracellularly, to oppose the signaling pathways of particular major hormones, with the glucocorticoid pathway being of particular relevance to PTSD and other stress-related disorders. (Figure 1)

This hormone-mediated upregulation of FKBP5 has been observed in the peripheral blood of depressed patients and controls treated with dexamethasone (a glucocorticoid receptor agonist), bronchial biopsies from asthmatic patients treated with inhaled corticosteroids, and mice fed corticosterone-treated drinking water – suggesting a broad and conserved response of this gene to glucocorticoid signaling. (Kelly et al., 2012; Lee et al., 2011; Menke et al., 2012)

A 2011 investigation using food deprivation and restraint stress in mice further demonstrated that specific brain regions demonstrated large changes in *Fkbp5* expression following these ethologically relevant stressors – with the paraventricular nucleus of the

hypothalamus and the amygdala demonstrating large increases in *Fkbp5* expression. (Scharf, Liebl, Binder, Schmidt, & Muller, 2011) These findings show that brain regions known to participate in stress- and anxiety-related behaviors harbor cells dynamically expressing *Fkbp5* in the mouse.

Subsequent epigenetic analyses of *Fkbp5* in human peripheral blood have yielded insight as to how stress may be encoded into the transcriptional memory of the gene. In a 2013 study, a region in intron 7 of *Fkbp5* showed reduced 5-methylcytosine (5mC) in those with the risk allele and with an increased score on the Childhood Trauma Questionnaire (CTQ). This variant allele, *rs1360780*, was chosen for its proximity to a GR element, a short DNA motif that binds GR. In these studies, the risk allele of *rs1360780* is A/T, and the protective allele is C/G. Klengel et al. (2013) showed that the risk allele enhances expression via GR mediated upregulation using a luciferase assay. This effect in the native *Fkbp5* gene is likely due in part to long range interactions between the intronic sequence and the promoter. Furthermore, GR-mediated de-methylation of this site is persistent in primary neuronal culture, and methylation of the CpG containing GRE in intron 7 blunted GR-mediated gene upregulation. (Klengel et al., 2013)

A 2011 study demonstrated that mice deficient in *Fkbp5* did not demonstrate any changes in behavior at baseline, but after sufficiently intense stressors, *Fkbp5* deficient mice demonstrated reduced corticosterone response, a greater time spent actively coping in the forced swim test, and a mild hypersensitivity of the GR. (Touma et al., 2011) Another functional study of *Fkbp5* occurred in Hartmann et al, 2012 – the authors used an *Fkbp5* knockout to assess the role this gene may play in mediating the effects of chronic social defeat. In this paradigm, the knockout animals demonstrated a less vulnerable phenotype – showing lower adrenal weight and basal corticosterone, and greater active stress-coping. These results suggest that in the absence of

Fkbp5, mice were able to achieve a more robust negative feedback of the HPA signaling axis. (Hartmann et al., 2012)

Memory and Epigenetics

Dynamic regulation of transcriptional processes in the nucleus govern the production of RNA. In short, DNA is packaged into a super-structure by histone proteins (together referred to as chromatin), and post-translational modification of these histones, as well as direct modification of the nucleotide base pairs, results in changes in the accessibility of DNA elements and the DNA-binding transcription factor repertoires that interact with those particular regulatory elements. (Figure 2)(Aguilar & Craighead, 2013) Broadly defined, these chromatin regulatory dynamics are referred to as epigenetics. One of the most-thoroughly studied epigenetic marks is DNA, cytosine methylation, or 5-methylcytosine (5-mC). Originally proposed to have transcriptional regulatory function in the 1970s, the dynamic nature of DNA methylation has been increasingly appreciated. Notably, it has recently been shown that DNA demethylation can occur dynamically in post-mitotic cells. The identification of the TET1 protein as a 5mC oxidizing enzyme, the recognition of further oxidized products (5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)), and finally the recognition that 5-carboxylcytosine can be excised by the thymine-DNA glycosylase base excision repair pathway, identified for the first time a replication-independent method of DNA demethylation. (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009)

However, while 5-hydroxymethylcytosine (5hmC) is clearly an intermediate of the demethylation process, it is also a stable mark – suggesting that it may play its own regulatory role. This was furthered by findings that all of the methylation intermediates (5hmC, 5fC and 5caC) appear to recruit distinct transcription factors. (Iurlaro et al., 2013; Spruijt et al., 2013) In

post-mitotic neurons in particular, 5hmC seems to result in a reduced capacity to bind methylcytosine binding protein 2 (MeCP2), a crucial mediator of methylation-directed gene silencing. Furthermore, this functional demethylation seemed limited to gene bodies, rather than intergenic-enhancer regions. (Mellen, Ayata, & Heintz, 2017) This suggests that there is an interplay between demethylation and the recruitment or repulsion of specific transcription factors, with 5hmC being a particularly stable intermediate.

With the discovery of a replication-independent cytosine demethylation pathway, it began to be appreciated that the abundance of 5hmC is particularly high in the brain, as compared to other tissues. Initially described to be quite prevalent in the Purkinje neurons of the cerebellum and in the forebrain, further tissue quantification studies confirmed 5hmC is abundant in a broad range of CNS tissues of mice and humans. (Globisch et al., 2010; Kriaucionis & Heintz, 2009; Nestor et al., 2012) This initial suggestion that 5hmC plays a particularly dynamic role in the central nervous system was confirmed first by a study showing neuronal activity dependent modulations in DNA methylation. (Guo et al., 2011)

DNA methylation changes with fear and memory processes

Differential methylation status has been associated with a variety of neurobiological outcomes, and manipulation of the key enzymes in the DNA methylation processing cycle have been causally linked with learning and memory. A few association studies have shown specific plasticity mediators undergoing dynamic methylation, such as *gadd45b* in neurogenesis (Ma et al., 2009) In other brain regions, 5-hmC dynamics have been linked with behaviorally relevant paradigms and are associated with plasticity and stress related genes, including the glucocorticoid receptor. (Li et al., 2015, 2016) In terms of stress, 5-hmC has been shown to be

disrupted in mouse models of autism, and prenatal stress (Dong et al., 2015; Papale et al., 2015) 5-hmC has also been shown to demonstrate sex-specific dynamics in response to stress. (Papale et al., 2016). In particular, it became clear that 5mC and 5hmC are actively regulated during neuronal activity and the correlation between memory and dynamic methylation is much stronger than between memory and a variety of histone modifications. (Halder et al., 2016) This is furthered by studies of mouse embryonic stem cells, in which it has been observed that 5hmC is the most-influential hub in the epigenomic communication network of mESCs – connecting DNA demethylation to nucleosome remodeling and key transcription factors in the pluripotency process. (Juan et al., 2016) These findings suggest that dynamic DNA methylation and 5hmC in particular play a central role in dynamic transcriptional processes.

Broadly, DNA methylation dynamics have been shown to be necessary for various types of memory consolidation. Initial evidence demonstrated DNA methyl transferase enzymes (DNMTs: specifically *Dnmt3a* and *3b)* were increased in the CA1 region of the hippocampus following contextual fear conditioning and inhibition of these enzymes immediately after training significantly inhibited fear expression, and presumably the memory consolidation process. Further work demonstrated that methylation dynamics in the anterior cingulate cortex are necessary for the long-term consolidation of contextual fear memories – linking systems level consolidation with DNA methylation. (C. A. Miller et al., 2010) The necessity of dynamic methylation was extended to appetitive tasks as well, via pharmacologic manipulation of DNMTs in the ventral tegmental area during a reward association task. (Day et al., 2013) In addition to the action of DNMTs in learning and memory, manipulations of Tet enzymes have demonstrated that 5mC oxidation is also critical for learning and memory. Tet1 knockout has been shown to influence activity dependent gene expression and memory extinction, and

hippocampal-specific Tet1 knock out inhibits the long-term consolidation of contextual fear memory. (Kaas et al., 2013; Rudenko et al., 2013) Furthermore, Tet1 knockout mice display a resistance to chronic restraint stress while Tet2 mice display a susceptibility to it. These findings, suggest a dissociable role of 5hmC dynamics in stress response. (Cheng et al., 2018) Specific to the amygdala, it has also been shown that DNMT activity is required for fear memory consolidation, fear memory reconsolidation, and reconsolidation of cocaine associated memory. (Alaghband, Bredy, & Wood, 2016; Jarome & Lubin, 2014; Maddox, Watts, & Schafe, 2014; Monsey, Ota, Akingbade, Hong, & Schafe, 2011; Shi et al., 2015) And finally, at an electrophysiological level, Dnmt as well as Tet function have also been shown to be necessary for glutamatergic synapse scaling, linking plasticity processes to methylation signatures. (Meadows et al., 2015)

Methylome Editing

Overall, the corpus of literature related to DNA methylation, learning, and stress, suggests that DNAm plays a crucial role in all of these processes in the CNS. However, the elucidation of specific gene pathways modulated during learning and the role that specific epigenetically active loci play in regulating these genes is not well understood. The current foundation of descriptive literature and broad manipulations have not yet demonstrated how specific epigenetic modifications influence genes of interest. Testing the causal relationships between particular epigenetic marks with chromatin dynamics, gene expression, cellular phenotypes, and behavior has recently been made possible with the advent of genome-editing technologies. Though still in its relative infancy, studies have demonstrated that a variety of genome-targeting proteins,

covalently linked with Dnmt or Tet enzymatic domains, have the capacity to alter methylation and gene expression in a targeted manner. (Liu et al., 2016; Maeder et al., 2013)

The goal of this thesis is to use convergent data from genome-wide studies of fear learning and human genetic association studies, to identify: (1) molecular components of fear memory consolidation in the mouse, (2) prioritize these gene pathways based upon stringent statistical criteria and human genetic association studies, and (3) test the causal associations between epigenetic regulation of these pathways and phenotype outcomes.

In the second chapter of this thesis, we seek to interrogate genome-wide transcriptome and methylome dynamics in the amygdala, to understand the types of regulatory pathways employed by the CNS during the fear memory consolidation process. Using these data, we identify targets based on convergent evidence from our rodent studies and human genetic association approaches – a crucial step to interrogate mechanisms underlying genes with conserved function in fear learning, that are likely contributors to human disease. In the third chapter of this thesis, we develop an animal model of stress-sensitized fear learning, to understand how *Fkbp5*, the gene that survived the selection criteria outlined above, is modulated by experience, a crucial aspect to understand the role of this gene given its role in the HPA axis. In the fourth chapter we employ methylome-editing via dCas9 constructs to test the causal relationship between the epigenetic regulation of *Fkbp5* and its expression.

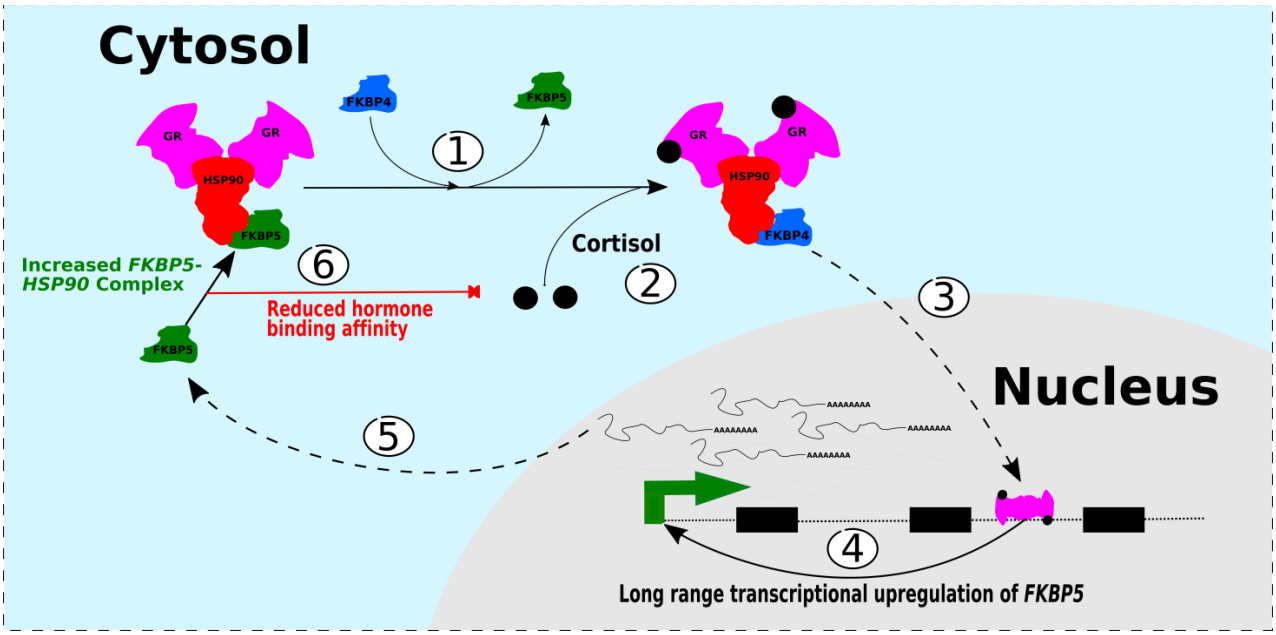

Figure 1. Ultrashort, intracellular negative feedback loop of HPA axis signaling. (1) Chemical equilibrium between *FKBP4-HSP90-GR* and *FKBP5-HSP90-GR* complexes exists within the cytosol. (2) Cortisol preferentially binds to *GR* in the *FKBP4-HSP90-GR* form, which possesses a higher cortisol binding affinity, is more stable, and promotes nuclear import. (3) Nuclear import of the complex allows (4) mature *GR* dimers to bind multiple genomic loci, including *FKBP5*. *GR* upregulates *FKBP5* transcription, in part, by binding intronic glucocorticoid receptor elements which form long range interactions with the promoter. (5) Increased *FKBP5* in the cytosol pushes the equilibrium towards the formation of *FKBP5-HSP90-GR*, (6) which has a reduced affinity for cortisol, thus decreasing overall *GR*-mediated epigenetic regulation.

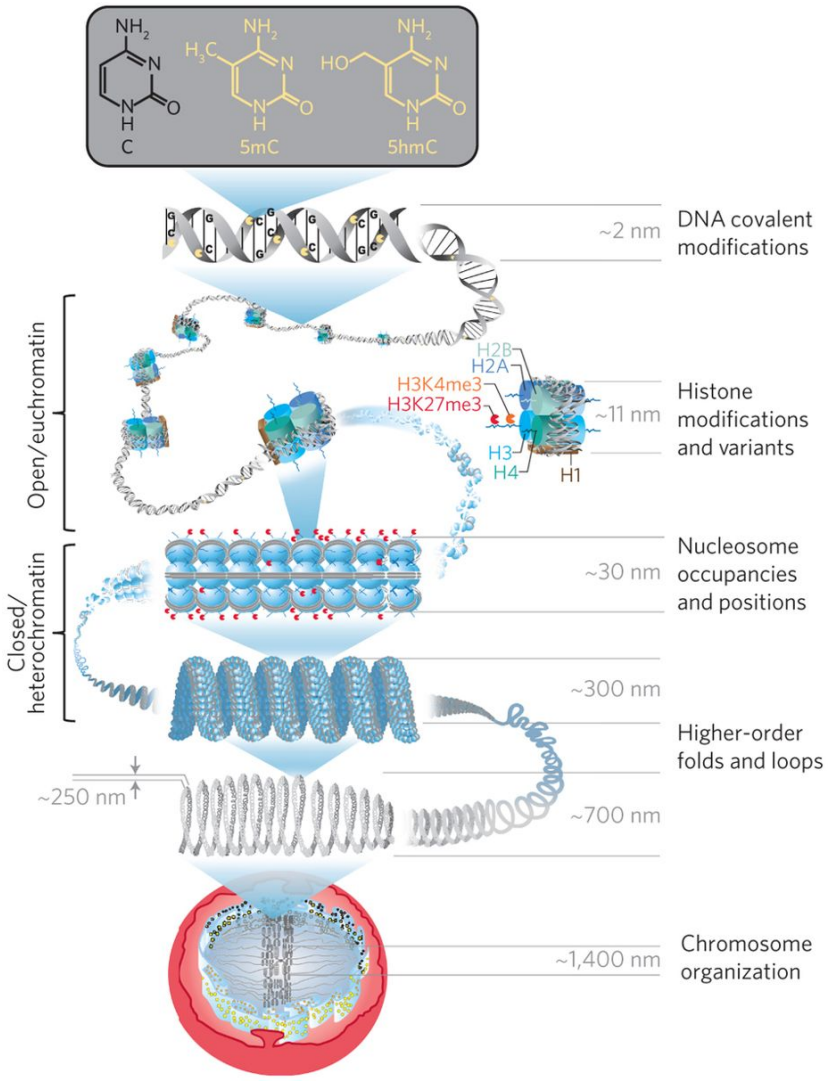

Figure 2. Schematic diagram of chromatin organization. Nucleotides can be covalently modified to produce bases with unique regulatory properties – the best understood of which is 5-methylcytosine (5mC) and its oxidized derivative, 5-hydroxymethylcytosine (5hmC). DNA is wound around histone protein octamers, which themselves can be post-translationally modified on "tail" structures which protrude from the histone-DNA complex. These nucleosomes can be organized into increasingly compact structures to facilitate biological processes such as transcription and cell division. *Reproduced from Aguilar and Craighead, 2013.* (Aguilar & Craighead, 2013)

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Chapter 2: Epigenetic and transcriptomic profiling of the BLA during fear memory consolidation

Introduction

Dynamic DNA methylation is a crucial process for the regulatory dynamics of gene expression and chromatin organization. Across biology, DNA methylation has been shown to be dynamic during development, and during a variety of biological processes, including stress, learning, and memory. (Dias, Maddox, Klengel, & Ressler, 2015) Much of the regulatory outcome of this dynamic DNA methylation potentially is due to alterations in transcription factor binding to DNA mediated by changes in these covalent cytosine modifications. Interestingly, in the brain, the presence of intragenic 5-methylcyosine (5mC) is negatively correlated with gene expression, and the density of 5-hydroxymethylcytosine (5hmC) is particularly high. This suggests that DNA methylation in the brain is particularly dynamic, (Luo, Hajkova, & Ecker, 2018) a result that has been dissected by the learning and memory community to reveal numerous associations between DNA methylation and learning.

DNA methylation dynamics have been shown to be necessary for various types of memory consolidation, in particular the consolidation of fear memories. Initial evidence demonstrated DNA methyl transferase enzymes (Dnmt) were increased in the CA1 region of the hippocampus following contextual fear conditioning and inhibition of these enzymes immediately after training significantly inhibited fear expression and the memory consolidation process. Further work demonstrated that methylation dynamics in the anterior cingulate cortex are necessary for the long-term consolidation of contextual fear memories – linking systems level consolidation with DNA methylation. (C. A. Miller et al., 2010) The necessity of dynamic

methylation was extended to appetitive tasks as well, via pharmacologic manipulation of Dnmts in the ventral tegmental area during a reward association task. (Day et al., 2013) In the amygdala, a brain region that coordinates much of the fear response, it has also been shown that DNMT activity is required for fear memory consolidation, fear reconsolidation, and reconsolidation of cocaine associated memory. (Alaghband, Bredy, & Wood, 2016; Jarome & Lubin, 2014; Maddox, Watts, & Schafe, 2014; Monsey, Ota, Akingbade, Hong, & Schafe, 2011; Shi et al., 2015) Taken together, these data suggest that the deposition of *de novo* 5mC is critical for neural function, and further work has clarified the role that the oxidation of 5mC to 5hmC has played.

In addition to the action of Dnmts in learning and memory, manipulations of the ten-eleven translocase (Tet) enzymes have demonstrated that 5mC oxidation is also critical for learning and memory. Tet1 knockout has been shown to influence activity-dependent gene expression and memory extinction, and hippocampal-specific Tet1 knock out inhibits the long-term consolidation of contextual fear memory. (Kaas et al., 2013; Rudenko et al., 2013) Furthermore, Tet1 knockout mice display a resistance to chronic restraint stress while Tet2 mice display a susceptibility to it, suggesting a dissociable role of 5hmC dynamics in stress response. (Cheng et al., 2018) And finally, at an electrophysiological level, Dnmt as well as Tet function have also been shown to be necessary for glutamatergic synapse scaling, linking plasticity processes to methylation signatures. (Meadows et al., 2015)

Global manipulations of DNA methylation drivers have elucidated the causal role of this modification in neuronal function. To clarify the specific genomic features that govern molecular dynamics involved in learning and memory we profiled 5hmC in the basolateral amygdala (BLA) during fear memory consolidation. We found widespread alterations in conditioned fear associated 5hmC, with an overlap between transcriptionally regulated genes, enhancers, CTCF

binding sites, and repetitive elements. We also find a genome-wide signature of reduced 5hmC in fear conditioned amygdala as compared to control.

Results

Approach to Combined Transcriptional and Epigenetic Profiling of the BLA During Fear memory consolidation

To identify the transcriptional and epigenetic pathways regulated during fear memory consolidation in the BLA we generated genome-wide datasets of mRNA and DNA 5-hydroxymethylation (5hmC) dynamics, 2 hours after fear conditioning. The selection of 2 hours post-conditioning was based on previous work from our group that has demonstrated that molecular changes necessary and sufficient for the expression of learned fear are dynamically expressed at this timepoint. (Andero, Dias, & Ressler, 2014; Andero et al., 2013; Chhatwal, Stanek-Rattiner, Davis, & Ressler, 2006; Dias et al., 2014; Heldt et al., 2014)

Mice either underwent auditory fear conditioning and were sacrificed 2 hours after the session, or were sacrificed from their home cage. (methods) Home cage mice, sacrificed within the same experimental session to control for day-to-day variability, were used as controls so that genes associated with learning and memory, as well as stress, could be identified. Flash frozen brain were sliced on a microtome, and tissue punches centered on the BLA were taken for mRNA-seq and 5hmC-seq. For both RNA-seq and 5hmC-seq, each sample consisting of pooled amygdala punches.

In general, the sequencing datasets were of good technical quality, with high quality per base and per sequence quality scores across all datasets. (Figure S1) The RNA-seq datasets demonstrated consistent variation in aggregate level FPKM calls and variance, with cluster

analysis of gene expression demonstrating a close relationship between home cage and fear conditioned replicates. (Figure S2) The 5hmC-seq HC and FC replicates demonstrated a high correlation of 5hmC density at each MACS peak indicating a consistency between experimental replicates. (Figures S3) Data quality control was performed by FastQC and reads were aligned with STAR. mRNA differential expression was calculated using the Cufflinks suite, and differential exon usage was calculated using the DEXSeq package. (Dobin et al., 2013; C Trapnell et al., 2013; Cole Trapnell et al., 2012a) 5hmC differential enrichment was calculated using two different methods.

Comparison of 5hmC-enrichment analyses: MACS vs MEDIPS

An open question is how best to identify differentially, epigenetically regulated loci in high throughput profiling experiments. We employed two popular approaches to assess the full range of differentially 5hmC-regulated regions. The Model-based Analysis for ChIP-Seq (MACS) package was used to identify 5hmC enriched regions (peaks) as compared to input, and peaks common to the replicates in one condition, and absent in the other condition, were considered a differentially hydroxymethylated region (MACS DHMRs). (methods) (Jianxing Feng, Liu, Qin, Zhang, & Liu, 2012) In parallel, the MEDIPS package was used to call differentially 5hmC regulated regions based on a 100 bp sliding window analysis (MEDIPS DHMRs). (Lienhard, Grimm, Morkel, Herwig, & Chavez, 2014) (methods) In general, many more MACS DHMRs were called, as MACS analysis determines enrichment by a local sliding window analysis as compared to input, and MEDIPS compares the home cage to the fear conditioned sample directly, correcting for multiple comparisons across the effective genome size.

We found the overlap between the two methods to be low - likely due to the higher stringency and genome-wide multiple corrections employed in MEDIPS. MACS identified 29,878 total DHMRs. MACS DHMRs were further prioritized by total difference in 5hmC density between home cage and fear conditioning. In short, ngsPlot was used to calculate the reads per kilobase million (RPKM) of 5hmC at each MACS peak, and peaks with a difference score greater than 0.1 were included in the final analysis. (methods) Finally, 18,287 MACS DHMRs with reduced 5hmC after fear conditioning and 1,992 with increased 5hmC after fear conditioning compared to home cage controls were kept in the final analysis. MEDIPS was more stringent in its identification of DHMRs, identifying 1918 significantly differentially 5-hydroxymethylated regions, with 1,384 reduced 5hmC regions after fear conditioning and 534 increased 5hmC regions after fear conditioning, at an adjusted p-value cutoff of 0.1, as has been used elsewhere in the literature. (Halder et al., 2016) The overlap between MEDIPS- and MACS-called DHMRs was low, with 55 shared DHMRs.

The unique approaches taken by each algorithm in determining differential methylation likely underlies the discrepancy between the two methods. As MACS does not employ a genome-wide correction to compare between conditions, but rather focuses on local sequencing background to determine enrichment, it may be that MACS identifies more sites with enriched methylation signatures. By contrast, MEDIPS compares the methylation density between conditions directly, and corrects across the genome. Thus, it may be that the sites identified as DHMR by MEDIPS are more likely to be strongly differentially hydroxymethylated as compared to MACS DHMRs, but conversely sites with enriched 5hmC but a smaller difference between conditions may not be picked up. However, both methods offered insight – as MACS was much more promiscuous, it overlapped significantly with genes significantly differentially regulated

during fear memory consolidation. And the further parsing based on 5hmC density allowed us to identify DHMRs that survived replication by targeted replication approaches we employ in chapter 4.

Differential Expression and Exon Usage in Fear memory consolidation

To elucidate the genes differentially regulated with fear conditioning, differential expression and exon usage were used as criteria from the RNAseq experiments. Using a statistical false discovery rate cutoff of 5%, 712 total genes were called as significantly differentially expressed, with 528 genes found to be downregulated in FC animals compared to HC and 184 genes upregulated. (Figure 1A) Using an FDR cutoff of 5%, we found 757 genes with differentially regulated exons. (Figure 1B). There was a significant overlap between differentially expressed genes and genes with differential exon inclusion; however, the majority of dynamic genes in either analysis were exclusively differentially expressed or containing differential exon usage. (Figure 1C)

We next examined the overlap between the genomic profiling (DHMR and RNAseq) modalities employed. We limited our analysis to cis-regulatory elements, limiting our definition of overlap to DHMRs within a gene body or a maximum of 10 kb upstream of the TSS, but stopping short if another gene inhabited those loci. In terms of gene expression differences, 240 genes were found to be common to differential exon usage and differential expression, while 472 genes were uniquely differentially expressed, and 518 genes uniquely exhibited differential exon usage. (Figure 1B, 1C) The overlap of MACS DHMRs with genic regions revealed 204 gene symbols who were both differentially expressed and methylated, and 241 gene symbols were found to exhibit differential exon usage.

Gene Expression is anti-correlated with 5hmC density at dynamic loci, but positively-correlated with whole-gene 5hmC density

Using the data at hand, we were curious as to whether 5hmC density and/or DHMRs significantly correlated with gene expression. Overall, the majority of differentially regulated genes displayed reduced expression with fear conditioning, and our annotation analysis indicates that promoter elements classically associated with gene expression display reduced 5hmC with fear conditioning (promoters and CpG classes) (Figures 1A and 2B). At a genome-wide level, 5hmC density was weakly positively correlated with gene expression in both home cage and fear conditioning (p-value $< 2 \times 10^{-16}$, $R^2 \sim 0.005$). (Figure S4.) This corroborates what others have found regarding genome-wide associations between 5hmC and gene expression. (Lin, Chen, & Hsu, 2017) However, when we analyzed the 5hmC density across gene bodies for genes exhibiting dynamic expression during fear conditioning, we observed that gene transcripts that were significantly upregulated displayed an overall reduction in 5hmC density, while gene transcripts that were downregulated did not exhibit any differences. (Figure 4 A, B)

Furthermore, when we examined the log-fold change of 5hmC in MEDIPS DHMRs contained within gene bodies of significantly regulated genes, and correlated these with changes in gene expression of those genes, we found a moderate negative correlation. Taken together, this suggests that loci with significantly increased 5hmC within gene bodies may, on average, drive reduced expression of transcripts. However, it may be that this is a specific consequence of 5hmC in gene bodies – a phenomenon that is prominent in the central nervous system. Another caveat is that we limited our analysis to cis-regulation, as we did not infer causal relationships

between distal DHMRs and gene expression, but focused only on intragenic methylation signatures.

This suggests that at any time point, the general correlation between gene body 5hmC and expression of a particular gene is positive, however, particular dynamic loci may in fact drive the overall negative regulation of gene expression. This could be through multiple potential mechanisms, including the recruitment of 5hmC specific transcription factors or changes in chromatin accessibility over repressive transcriptional elements.

Genomic Annotation of DHMRs

We next examined the classes of genomic elements that overlapped amygdala-specific DHMRs to gain insight into functionality, using the *annotatr* package for R. (Cavalcante & Sartor, 2017) (methods) Annotation of MACS DHMRs revealed that the majority of peaks were in intronic regions, intergenic sites, and over exons (Figure 2A) (see Table S1 for a count of DHMRs within each category). In terms of total number of peaks, interestingly, we found many fewer in fear conditioning as compared to home cage. This is indicative of a gross reduction in 5hmC with fear conditioning. This has been observed in other brain regions in animal models of stress as well. (Cheng et al., 2018) Log fold changes over MEDIPS DHMRs revealed that most genic regions displayed or trended towards reduced 5hmC with fear conditioning: promoters, intron-exon boundaries, exon-intron boundaries, and UTRs. Interestingly, most gene-proximal CpG elements (CpG islands, shelves, and shores) exhibited reduced 5hmC, while intergenic CpGs displayed increased 5hmC (Figure 2b). Using the *annotatr* package, single DHMRs can be annotated to multiple classes – reflecting the multiple classes of functional assignment that can be put on dynamic loci.

Exonic CTCF sites have been shown to mediate exon inclusion – suggesting that a lot of the differential splicing we are seeing may in part be regulated by changes in CTCF binding to exons, with concordant alterations in 5hmC status.

Genome-wide 5hmC is reduced, but repetitive and mitochondrial DNA loci demonstrate markedly increased 5hmC during fear memory consolidation

The difference in the number of MACS DHMRs demonstrating reduced 5hmC with fear conditioning suggested a large reduction in 5hmC occurring genome-wide. To explore this, we used a binned RPKM approach to ask whether 5hmC density is significantly changed at a genome-wide level with conditioning. We created 3 primary bins: one of the primary mouse chromosomes (1-21, X, and Y), one of the unannotated contigs present in the mouse (chrUn and contigs localized to chromosomal positions), and mitochondrial DNA. The 5hmC-seq reads were aligned to include only uniquely aligned reads and to randomly assign reads to only one multimapped loci (methods), in order to avoid bias induced by repetitive DNA sequences. We found that the main chromosomes displayed an overall reduction in 5hmC with fear conditioning, consistent with the reduction in MACS DHMRs, which only included these chromosomes. (Figure 3a) Surprisingly, however, the unannotated regions showed much higher densities of 5hmC with fear conditioning as compared to home cage. While it has been known that many of the reads obtained from MeDIP-seq experiments tend to localize at repetitive regions, this large increase, under identical alignment conditions, with fear learning was unexpected. (Figure 3b, S3) (Halder et al., 2016) The mitochondrial DNA also demonstrated very large increases in 5hmC, suggesting that mitochondrial 5hmC may have a functional role in

the production of gene products required for oxidative phosphorylation mediating cellular activity and metabolism. (Figure 3C)

Gene Set Enrichment Analysis Identifies enrichment of cellular energetics, protein production and modification, and heat shock pathway as regulated molecular processes in fear memory consolidation

As a final layer of analysis, we sought to improve our understanding of the functional classification of genes that are regulated at the transcriptional level during fear memory consolidation. Initially, using over-representation analysis we asked whether any GO Molecular Pathways were significantly regulated during fear memory consolidation.

We employed Gene Set Enrichment Analysis (GSEA), a functional class scoring pathway analysis algorithm to clarify the pathways enriched during fear conditioning as compared to home cage. (McLean et al., 2010; Mootha et al., 2003; Subramanian et al., 2005) We used the GO Annotation database, molecular function annotations, downloaded from Ensembl BioMart version 87. (Ashburner et al., 2000; Carbon et al., 2017; Zerbino et al., 2018). We found 29 pathways differentially enriched in fear conditioning and 1 pathway enriched in home cage as compared to fear conditioning. (Table 1) Given the overlap of gene annotations among annotated pathways, we employed the cytoscape program to identify relatedness among the significantly enriched pathways and manually annotated the grouped pathways. (Doncheva, Assenov, Domingues, & Albrecht, 2012) Pathways related to mitochondrial function and oxidative phosphorylation were the most coordinately enriched during fear conditioning, in addition to the Heat Shock Protein Binding GO pathway and pathways related to protein folding and translation. (Figure 5)

This upregulation of oxidative phosphorylation pathways is consistent with a plethora of literature demonstrating the dynamic role of mitochondria during neural plasticity. Mitochondria are actively trafficked during neural activity to support neuronal function and plasticity. They also have been shown to release and sequester Ca^{2+} from the cytosol - evidence points to uptake during activity and slow release over time afterwards to support LTP. These findings suggest that mitochondrial energy production is critical, not only for a proper transmitter release *via* vesicle exocytosis, but also for mobilization of reserve synaptic-pool vesicle and regulation of synaptic strength. The slow mitochondrial efflux of calcium results in a minutes-lasting plateau of calcium concentration, which in turn causes facilitation of the synaptic response. Previous work demonstrated that blocking the mitochondrial calcium uptake resulted in a transient increase in presynaptic calcium levels and impaired neurotransmission during intense stimulation. (Todorova & Blokland, 2017; Williams, Thompson, Mason-Parker, Abraham, & Tate, 1998) Taken together, this suggests that neural plasticity associated with fear learning in the BLA is coordinately upregulating pathways for oxidative phosphorylation. Furthermore, at the level of methylation, the large increase in mitochondrial DNA 5hmC suggests that intramitochondrial genes necessary for oxidative phosphorylation are also being regulated. (Figure 3C) Furthermore, genomic pathway analysis of MACS-DHMRs revealed the top regulated pathway in DHMRs with reduced 5hmC during fear conditioning to be in the “Genes involved in the integration of energy metabolism.” (Figure S5-F)

Another set of pathways found to be coordinately enriched in fear conditioning were the GO pathways for Heat Shock Protein binding and its downstream pathway, HSP90 protein binding. The heat shock response is a well-characterized cellular consequence of many types of stress, including even psychosocial stress and the HPA axis response – wherein components of

the HSP90 protein complex, in particular, have been shown to regulate many diverse steroid hormone responses, including the response to corticosterone. Taking a deeper dive into this pathway, we charted out the interrelated genes in this set, and identified pathways containing significantly differentially expressed genes. (Figure 6) Within this upregulated pathway, we found 7 genes which also were individually identified as significantly upregulated with fear conditioning, suggesting that these genes are acting as the primary drivers of upregulation in this pathway. To see if any of these key nodes were 5hmC regulated, we took the overlap of the MACS-called 5hmC peaks with these genes and found *Fkbp5* to contain two DHMRs demonstrating reduced 5hmC with fear conditioning at a MACS FDR of 5%.

Transcriptional and Epigenetic regulation of the Heat Shock pathway and Fkbp5 during fear memory consolidation

FKBP5 gene regulation and methylation, in particular, has been shown to be significantly associated with stress and Post-traumatic Stress Disorder, as well as broader stress-related psychopathology. (Bevilacqua et al., 2012; Boscarino, Erlich, Hoffman, & Zhang, 2012; Collip et al., 2013; Roy, Gorodetsky, Yuan, Goldman, & Enoch, 2010; Zannas & Binder, 2014) Variants located in the glucocorticoid receptor elements of *Fkbp5* have been shown to predispose individuals who undergo early life stress to future risk for PTSD, as well as depression. (Appel et al., 2011; Binder et al., 2008; Lavebratt, Aberg, Sjoholm, & Forsell, 2010; Zimmermann et al., 2011) In addition, variants in FKBP5 have been shown to modulate response to antidepressant treatments. (Binder et al., 2004)

Thus, the *Fkbp5* gene appears to be strongly associated with a variety of translational outcomes, and in our screen for epigenetically regulated pathways associated with fear

conditioning, we find *Fkbp5* to be an unbiased candidate. We first sought to validate the transcriptional and methylation dynamics at the *Fkbp5* gene. In an independent cohort of fear conditioned mice, we performed fear conditioning, as well as a tone-alone control to assess the impact of novel environmental stress on gene transcription, and also measured *Fkbp5* RNA transcript levels. We found *Fkbp5* to be significantly upregulated compared to home cage control, replicating our original RNA-seq finding. We also found *Fkbp5* to be significantly upregulated in fear conditioning compared to the tone alone control, further supporting an association of this gene with the fear memory consolidation process. (Figure 7B) To validate the methylation level findings, we performed targeted bisulfite sequencing of the DHMRs in *Fkbp5*. We found that one of the DHMRs replicated a reduced methylation signature during fear conditioning, consistent with our genome-wide screen. (Figures 7A, C). Interestingly, the significantly reduced methylation was observed in CHH context cytosines that exhibited ~10% reductions in methylation.

Discussion

Our data shine a light on the specific genomic consequences of DNA methylation dynamics in the amygdala. We find that not only does 5hmC change in gross density with fear learning, we also find a strong overlap between gene regulatory elements (enhancers, CTCF binding sites), and an unexpected large modulation of 5hmC at repetitive elements and in mitochondrial DNA, suggesting a functional role for dynamic DNA methylation in these less genomic contexts. At the level of the coding genome, we find a strong negative correlation between dynamic, intragenic 5hmC and gene expression, which is contrary to genome-wide estimates of 5hmC and gene expression that we also replicate in our study.

We identified several manually curated modules of gene expression pathways. Several were intuitive: an upregulation of oxidative phosphorylation pathways, myelin sheath producing pathways, protein folding pathways, and transcriptional pathways. These were expected as an abundance of literature has demonstrated the necessary role that mitochondrial dynamics, protein translation, and gene transcription processes play in the plasticity process. (Davis & Squire, 1984; Milner, Squire, & Kandel, 1998; Todorova & Blokland, 2017) Our focus shifted to the heat shock pathway, which contained a number of genes that had previously been implicated in stress and anxiety disorders in genetic association studies in humans.

The strong overlap between mitochondrial DNA and 5hmC was surprising. But given the large activation of pathways of oxidative phosphorylation we observe in our GSEA analysis, it is not surprising that the suite of proteins encoded within the mitochondrion for energetics would also be mobilized. The very large density of 5hmC we observe is likely a result of the massive number of mitochondria present in any one cell – with estimates of over 100 mitochondria per synaptic junction, suggesting that each neuron possesses thousands to hundreds of thousands of mitochondria. (Misgeld, Kerschensteiner, Bareyre, Burgess, & Lichtman, 2007) Indeed, a 2017 study found that directing methyltransferases to the mitochondrial genome reduced the expression of genes if the GpC context cytosines were preferentially targeted. (van der Wijst, van Tilburg, Ruiters, & Rots, 2017) This suggests that perhaps 5hmC also plays an important role in demethylating and activating the crucial genes in the oxidative phosphorylation pathways encoded within mitochondria. (Shadel, 2008)

The overlap between repetitive DNA sequences and DNA methylation has been previously reported; however, the dynamic nature of 5hmC at these repetitive loci suggests that dynamic regulation of repetitive DNA elements may be a feature of learning and memory related

processes. (Halder et al., 2016) As with the mitochondrial DNA analysis, the quantity of repetitive elements is not fully accounted for, and reference genomes can only represent a unique copy, so alignment to these widespread elements can appear highly concentrated, when the biological reality is that these elements are dispersed throughout the genome. 20-80% of the eukaryotic genome is estimated to be occupied by transposable elements. Nonetheless, the dynamic modulation of these repetitive elements suggests that RNAs within these elements, or cis/trans regulatory functions of these loci are being activated by fear learning. Indeed, transposable elements have been shown to be quite dynamic; to name a few examples, they are: actively transcribed, activated during aging, play a role in defining stem-cell states, and are drivers in cancer progression. (Faulkner et al., 2009; Kelley & Rinn, 2012; Li et al., 2013; Lu et al., 2014; Ohnuki et al., 2014; Shukla et al., 2013; Wang et al., 2014) Interestingly, studies have shown that transposition in the neural genome can be quite broad, with high frequency transposition during development, some transposition during neurogenesis, reports of increased transposition in schizophrenia, and increased transposition of L1-Line elements that occurs with early life stress in mice. (Bedrosian, Quayle, Novaresi, & Gage, 2018; Bundo et al., 2014; Li et al., 2013; Thomas, Paquola, & Muotri, 2012) Taken together these findings suggest that transposition is dynamic across normal and disease biology, and transcription of these elements may also play an active role in the normal neuronal plasticity process, suggesting an intersection between these phenomena that is not well understood.

Finally, we demonstrate using gene set enrichment analysis the functional gene modules activated during the fear memory consolidation process. The modular nature of biological systems has been appreciated for decades, and our ability to statistically identify relevant gene categories for specific processes has been growing with the development of novel algorithms to

analyze unbiased, whole genome datasets. (Hartwell, Hopfield, Leibler, & Murray, 1999) We identified several categories of gene function altered during fear memory consolidation, but our focus shifted to the regulation of the heat shock pathway. A category of genes for which a multitude of human, genetic association studies have pointed towards as being nodes where variation between individuals associates with psychopathology – particularly, stress-related psychopathology. The *Fkbp5* gene is a central player in regulating the sensitivity of the heat shock complex to hormonal signaling, and in particular, its epigenetic state has been linked to the history of trauma experienced by individuals. (Klengel et al., 2013) Herein, we demonstrate that this particular gene is regulated by dynamic methylation in the amygdala, and its expression can be causally manipulated by targeted methylation and demethylation.

One shortcoming of this study and all bulk tissue studies is that the contribution of individual cell types cannot be disentangled easily. It is possible that subsets of the dynamic gene and genomic elements modulated by 5hmC are specific to particular cell types. Single-cell technologies and follow up studies of DNA methylation in learning and memory will further identify the cell-type specific regulation of these elements.

Methods

Animals

All experiments were performed on adult, male, 10 week old, wild-type C57BL/6J mice received from Jackson Labs. Mice were group-housed in a temperature-controlled vivarium, with

ad libitum access to food and water. Mice were maintained on a 12-h light/dark cycle (dark from 7 pm – 7 am), with all behavioral procedures being performed during the light cycle in the morning, between 8 am -10 am. All procedures used were approved by the Institutional Animal Care and Use Committee (IACUC) of Mclean Hospital and in compliance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Fear conditioning and tissue collection

Data obtained for the auditory fear conditioning RNA-seq was previously published and methods for the generation of these mice can be found there. (Lori et al., 2018) Mice used for 5hmC-sequencing were generated using identical behavioral parameters. In short, mice were habituated to white-light illuminated, standard rodent modular test chambers 10 min on 2 consecutive days prior to fear conditioning. Fear conditioning consisted of five trials of a novel tone conditioned stimulus (CS; 30 s tone, 6 kHz, 70 dB), which co-terminated with a foot-shock (500 ms, 0.6 mA) unconditioned stimulus (US). The Pre-CS period lasted 180 s and a variable inter-trial interval (ITI) was used between each CS-US pairing to result in a total conditioning session which lasted 840 s. The apparatus was cleaned with Quatricide® after each mouse.

All mice were sacrificed either under basal conditions or 2 hours after fear conditioning with brier isofluorane exposure and decapitat. Brains were immediately frozen on dry ice and stored at -80ºC. Samples were subsequently sliced on a microtome (Leica Biosystems SM 2000R) and 1 mm micro punches centered over the BLA were taken. Punches for 2 mice of the same home-cage and behavioral condition were pooled together into each sample.

RNA isolation, library prep and sequencing

This protocol was carried out as described in Lori, et al, 2018. (Lori et al., 2018) RNA extraction, QC, library preparation and sequencing were conducted by the Yerkes Non-Human Primate Genomics Core (Atlanta, GA). In short, total RNA was isolated and purified from each sample using a bead milling homogenizer and the miRNeasy Mini kit (Qiagen cat: 217004). RNA quality and quantity were verified with the Agilent 2100 BioAnalyzer PicoChip (Agilent Technologies) before sequencing. Libraries were prepared using the Illumina TruSeq RNA kit(Illumina cat: RS-122-2001). Briefly, 250 ng of total RNA was used for library preparation. The final library was validated using a High Sensitivity DNA chip on the Agilent Bioanalyzer. The libraries were further quantified on the Qubit 2.0 Fluorometer using the High Sensitivity dsDNA assay (Life Technologies cat: Q32851). Libraries were normalized, cluster generation was performed on the V3 flowcell on the Illumina cBot, and the clustered flowcell was sequenced on the Illumina HiSeq1000 system using a paired-end 101 cycles run. PhiX spike-in was used as an internal control.

5-Hydroxymethylcytosine Immunoprecipitation Sequencing

5-hydroxymethylcytosine capture and high throughput sequencing were carried out as previously described. (Cheng et al., 2018) Genomic DNA was isolated from pooled, (2 mice, bilateral BLA) frozen punches by overnight incubation in 600 µL digestion buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) and Proteinase K (Thermo Fisher, cat# EO0491) at 55°C. The next day, a Phenol:Chloroform:Isoamyl Alcohol (25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA) (Sigma-Aldrich, cat# P-3803) extraction was carried out, with the final DNA pellet was washed with 75% ethanol, air-dried, and eluted with Nuclease-Free Water (Ambion).

5hmC enrichment was carried out using a modified selective chemical labeling method and libraries were generated using 25–50 ng of input genomic DNA or 5hmC-captured DNA to initiate the protocol. Libraries were produced using the Illumina protocol for Preparing Samples for ChIP Sequencing of DNA" (Part# 111257047 Rev. A). All sequencing libraries were run on Illumina Hi-seq 2000 machines using a single end 50 bp run.

Targeted Bisulfite Sequencing

In brief, genomic DNA was isolated as described above and the EZ DNA Methylation-Gold kit from Zymo Research was used to bisulfite convert genomic DNA. (Zymo cat: D5005) Primers were designed to amplify from bisulfite converted DNA using the MethPrimer2 web tool. (L.-C. Li & Dahiya, 2002) In short, primers were designed to produce amplicons between 100 – 300 bp long. Default parameters were used except product CPGs were set to 0, and in a subset of primers degenerate bases were used if a primer could not otherwise be designed. (Table S1 : primer sequences) The resulting bisulfite amplicons were then made into libraries for sequencing using the NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina. (NEB cat: E6240L) 2-5 PCR cycles were used to amplify the final libraries, which were quantified by Qubit 2.0 Fluorometer using the High Sensitivity dsDNA assay (Life Technologies cat: Q32851), prior to sequencing on the Illumina Miseq (Model M00764). Libraries were sequenced using the V2, 300 cycle reagent kit, using a paired end protocol (Illumina cat: MS-102-2002)

Genomic Alignment with STAR

As with all downstream analyses, the mm10 (GRCm38) version of the mouse genome was used (Waterston et al., 2002). Alignment to the mm10 UCSC Mouse Assembly was performed using STAR (version 2.5.3). (Dobin et al., 2012) The mm10 star index was produced

using the mm10 reference sequence (date modified: 02/07/2012), and the most recent GTF file downloaded from UCSC genome browser FTP server and Table Browser, respectively. The NCBI RefSeq designations for gene names was downloaded. (Karolchik et al., 2004; O'Leary et al., 2016) The "sjdbOverhang" option was set to 99 for RNA-seq data and 49 for 5hmC-seq data. Alignment was carried out using standard options, but for the RNA-seq data with "outSAMstrandField" set to intronMotif and "outFilterIntronMotifs" set to RemoveNoncanonical for downstream cufflinks analysis.

Quality Control of Sequencing Data:

Fastq files were analyzed by FastQC to determine quality scores and to survey general read characteristics. (Krueger et al., 2010) Cutadapt 1.4.2 was used to trim sequencing low quality base calls and remove adapter sequences. (Martin, 2011) Cutadapt was used with options -minimum-length 20 and –quality-cutoff 20. MultiQC was used to aggregate FastQC analyses and generate plots. (Ewels, Magnusson, Lundin, & Kaller, 2016)

Cufflinks differential expression analysis

STAR aligned files and the same GTF used in alignment were used to calculate differential gene expression using the cuffdiff module of the cufflinks suite (version 2.2.1). (Cole Trapnell et al., 2012b) The false discovery rate (FDR) was controlled at 5% to account for multiple testing in all analyses ($q<0.05$).

Identification of MACS DHMRs.

One method we used to identify differentially 5-cytosine hydroxymethlylated regulated regions between samples was the Model Based Analysis of ChIP-seq (version 1.4.0), a Poisson-based peak identification algorithm (MACS DHMRs). (J Feng, Liu, Qin, Zhang, & Liu, 2012) MACS DhMRs were determined by comparing each sample to input, removing duplicate reads, and selecting regions called as DHMRs in all replicates, with an overlap between peaks > 1 bp. Default options for the mouse genome and p-value were used, and peaksplitter was used to call subpeaks as DNA methylation peaks are often broadly distributed as compared to transcription factor ChIP-Seq.

Identification of MEDIPS DHMRs.

The second method we used to identify differentially 5-cytosine hydroxymethlylated regulated regions was the R package MEDIPS (version 1.16.0), a sliding-window analysis based on read counts in a fixed window size. We used the mm10 BSgenome, included only unique reads while replacing multimapped reads with one representative read, and set a window size of 100 bp, in order to call highly statistically significant windows. Downstream of enriched peak calling, windows with > 101 bp overlap were merged to identify large DHMRs. A 10% FDR correction criteria was used to call significant DHMRs

NGS Plot for aggregate 5hmC Plots

Aggregate 5hmC plots were created using ngs plot. (Shen, Shao, Liu, & Nestler, 2014) Options used included a moving window of width 1 to smooth the average profile, 95%

confidence interval was included into the aggregate gene plots to estimate sample variance and significance, and fragment length was set to 300.

Analysis of Targeted Bisulfite Sequencing

The BISMARK package was used to analyze the targeted bisulfite sequencing datasets. (Krueger & Andrews, 2011) A bisulfite converted genome was produced using the mm10 genome, and counts of methylated and unmethylated cytosines were generated from paired end, 150 bp reads using Bowtie2. (Langmead & Salzberg, 2012) Differentially methylated cytosines were determined in R using Student's T-Test and FDR correction based on the number of cytosines tested in a bisulfite amplicon.

DHMR-based Pathway Analysis: GREAT

To identify GO annotations, MSigDB pathways, and MSigDB transcription factor motifs associated with DHMRs, we used GREAT, a web-based program that annotates non-coding regions of the genome largely through the annotation categories of proximal genes. (McLean et al., 2010) The mm10 genome was used, with the Basal plus extension option selected, with proximal distances set to 5 kb upstream, 1 kb downstream, and distal gene annotation distance set to 1000 kb. Curated regulatory domains were included in the analysis.

Genic Assignment of DHMRs with Annotatr

To understand the genomic context of each DHMR, the R package Annotatr was used to identify the categories of CpG patterns (CpG islands, shores, and shelves), Fantom enhancers,

and intragenic elements (promoters, exons, introns, and UTRs) represented by each DHMR. (Cavalcante & Sartor, 2017) Quantification and display of these annotations was carried out in R.

GSEA for Gene-based Pathway Analysis

In order to understand the gene pathways differentially regulated by fear conditioning, we used the Gene Set Enrichment Analysis tool. (Subramanian et al., 2005) We used "fear conditioning" or "home cage" as binary phenotype measures and used per sample, FPKM measures derived from cuffdiff to rank genes. GO biological process gene sets were downloaded from MSigDB (version 5.2) and converted to the mouse orthologues. Permutations (1000) of phenotype labels were used to generate a family-wise error rate (FWER) for each GO term. A stringent FWER of < 0.25 was the significance cutoff for inclusion, as per the recommendations of the developers (See the GSEA frequently asked questions section https://software.broadinstitute.org/cancer/software/gsea/wiki/index.php/FAQ for more details).

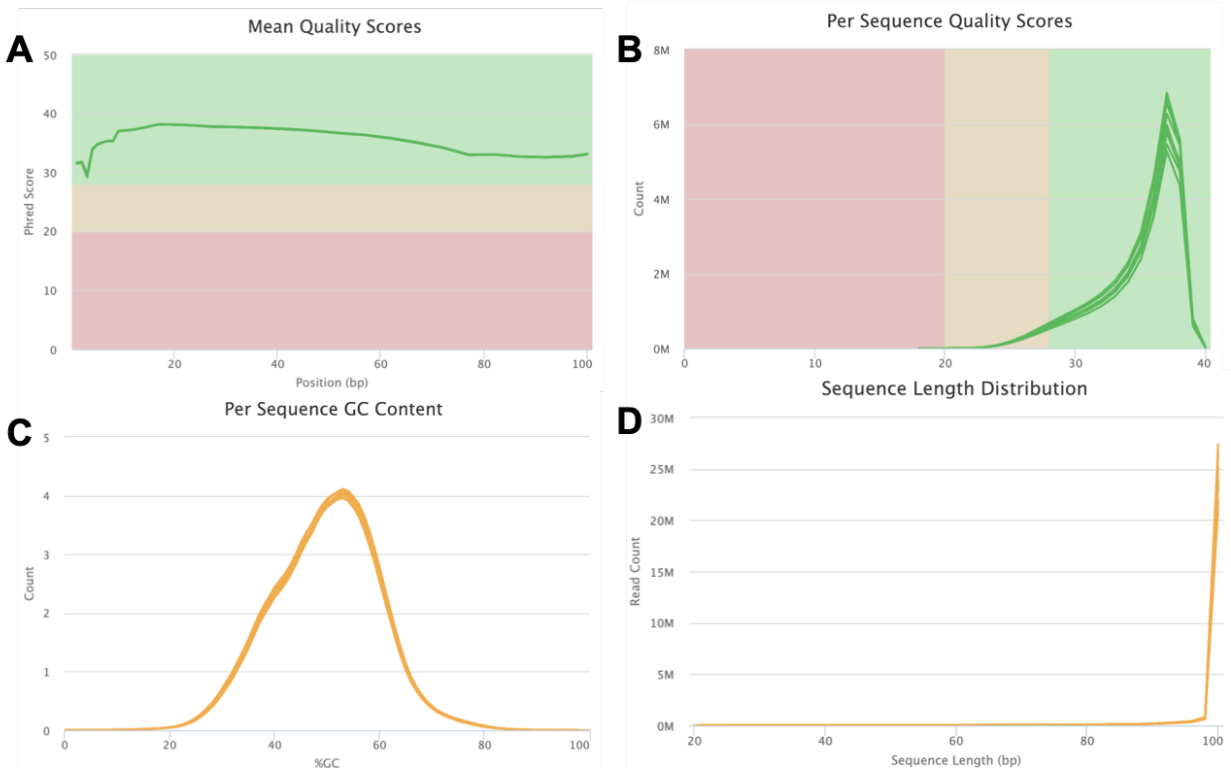
Cytoscape for the Visualization of Pathways

Cytoscape (version 3.7.0) was used to visualize the relatedness among significant GO terms, as well the network of genes represented within particular pathways. (Shannon et al., 2003) Within cytoscape, the enrichment map plug in was used to import GSEA results and style options within the program were used to manually annotate inter-related gene clusters (Merico, Isserlin, Stueker, Emili, & Bader, 2010)

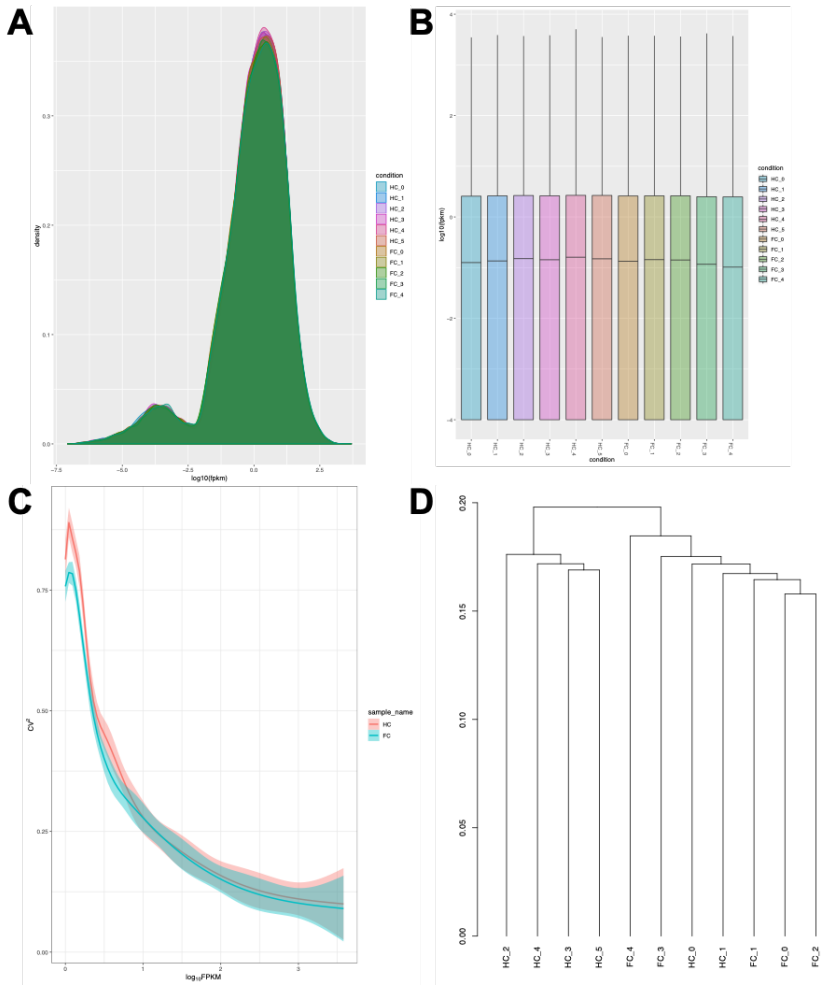
Miscellaneous methods

Tables created with stargazer package for R. (Hlavac, 2018)

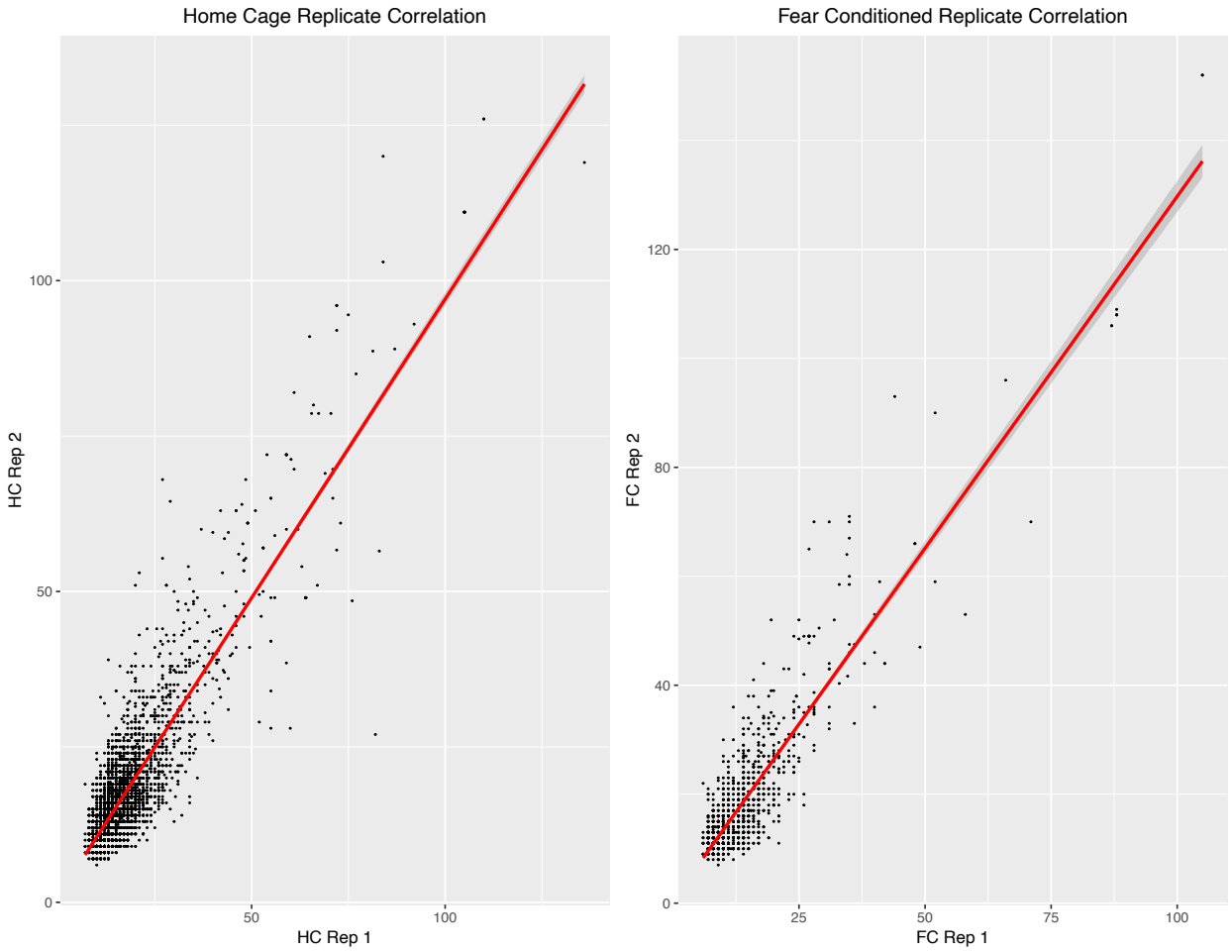
Tables and Figures



Supplementary Figure 1. Aggregate Plots of quality control analysis of RNA-seq fastq output files. Fastq files were analyzed by FastQC and data was combined with MultiQC to produce aggregate plots. (A) Aggregate per base quality scores; (B) mean quality score per sequence; (C) GC content per sequence; (D) Sequence length distribution.



Supplementary Figure 2. Quality control analysis of RNA-seq Cufflinks output files. Cuffdiff output was analyzed with CummeRbund for (A) Sample-by-sample expression level density by histogram and by (B) barplots, and (C) coefficient of variation by condition (Home Cage vs Fear Conditioned), and (D) a dendrogram of the distance between samples.



Supplementary Figure 3. Correlation between replicates for 5hmC-sequencing datasets. Home cage correlation had an adjusted $R^2 = 0.7394$ (p-value $< 2.2 \times 10^{-16}$) and the fear conditioned correlation had an adjusted $R^2 = 0.8138$ (p-value $< 2.2 \times 10^{-16}$).

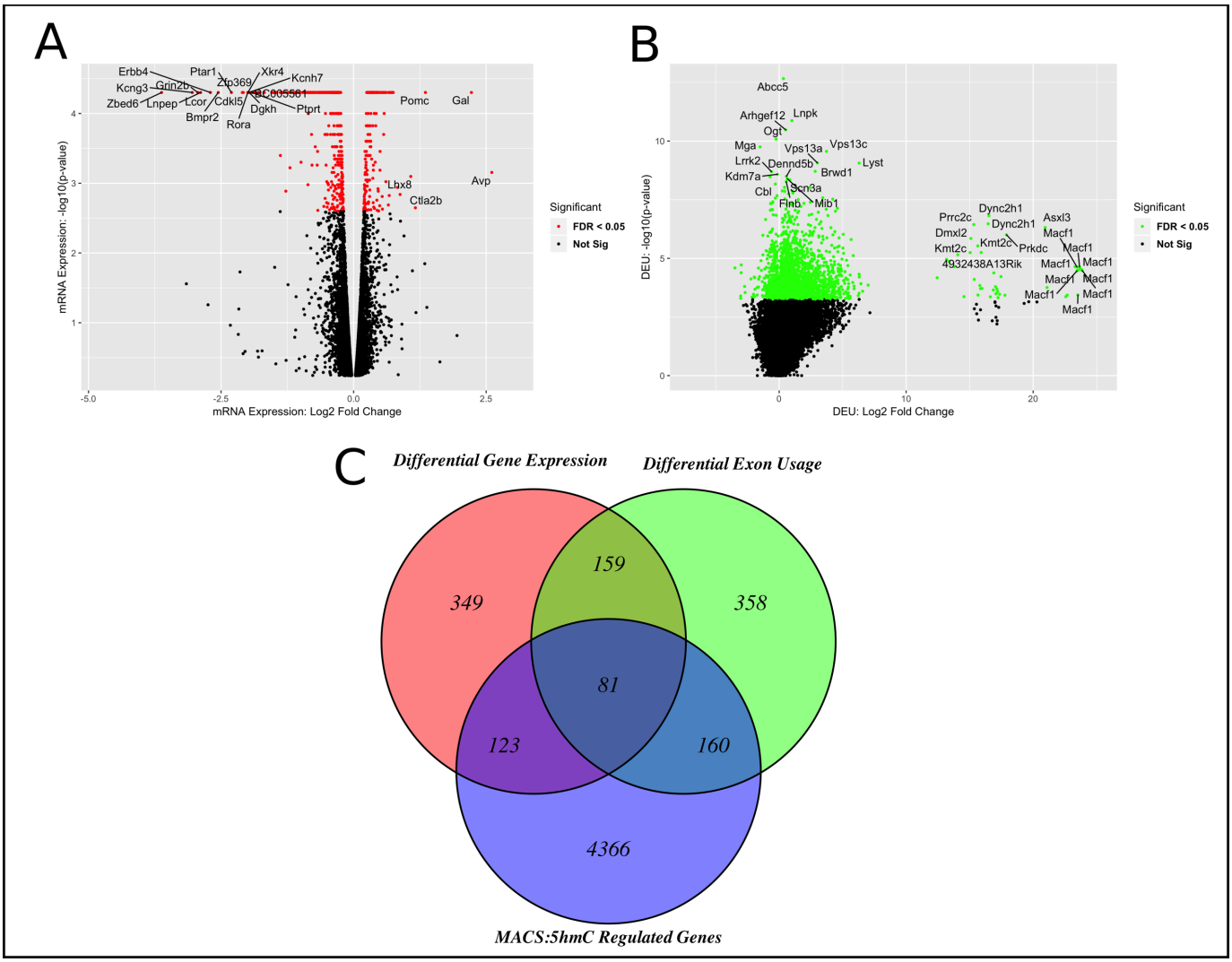


Figure 1. Volcano Plots of molecular dynamics arising 2 hours after fear conditioning. (A) Differentially expressed genes (FDR correction of 5%); genes were labelled sparsely based on log base 2-fold change cutoffs (greater than 0.7 or less than -2). (B) Genes containing differentially expressed exons (FDR 5%). (C) Overlap between differentially expressed, differential exon usage, and 5hmC at the level of gene annotation.

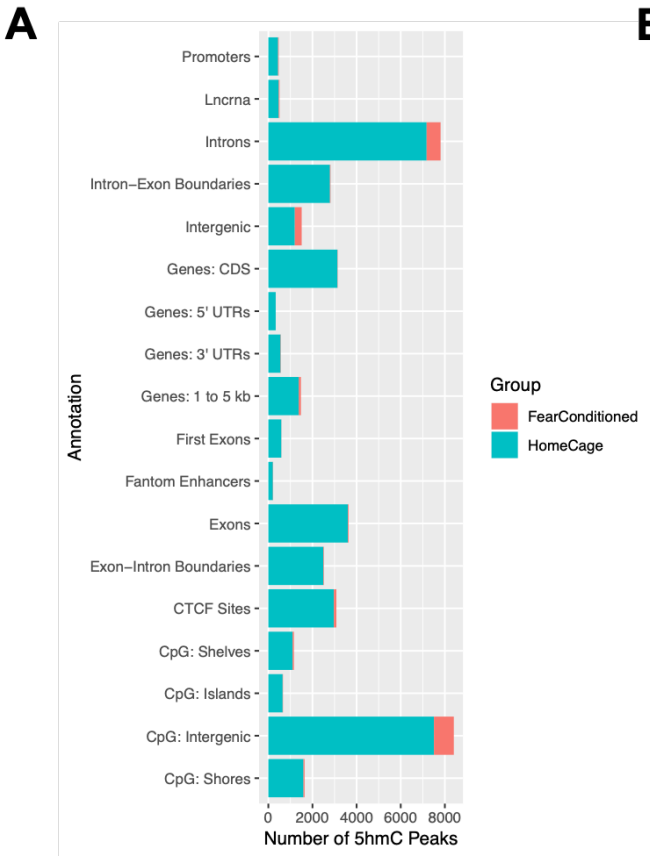


Figure 2. (A) Annotations and peak enrichment of DHMRs called by MACS. Bar sizes represent total number of DHMRs identified in each category, with blue shading representing DHMRs with high 5hmC during home cage and red representing DHMRs with high 5hmC during fear conditioning. (B) Annotations and log fold change of high confidence MEDIPS DHMRs. Bars represent log base 2 change in 5hmC density at DHMRs called with a q-value cutoff of 0.1

Annotation Count of MACS Peaks

Annotation	Peaks Inc 5hmC	Peaks Dec 5hmC
CpG: Intergenic	1292	15324
CpG: Islands	4	1011
CpG: Shelves	55	2762
CpG: Shores	69	3560
CTCF Sites	145	7444
Fantom Enhancers	20	442
Genes: 3' UTRs	13	1407
Genes: 5' UTRs	2	606
Exons	60	8447
Intergenic	345	1879
Introns	1034	16177
Promoters	28	920
Lncrna	86	1098

Table S1. Counts of MACS peaks in each annotation class

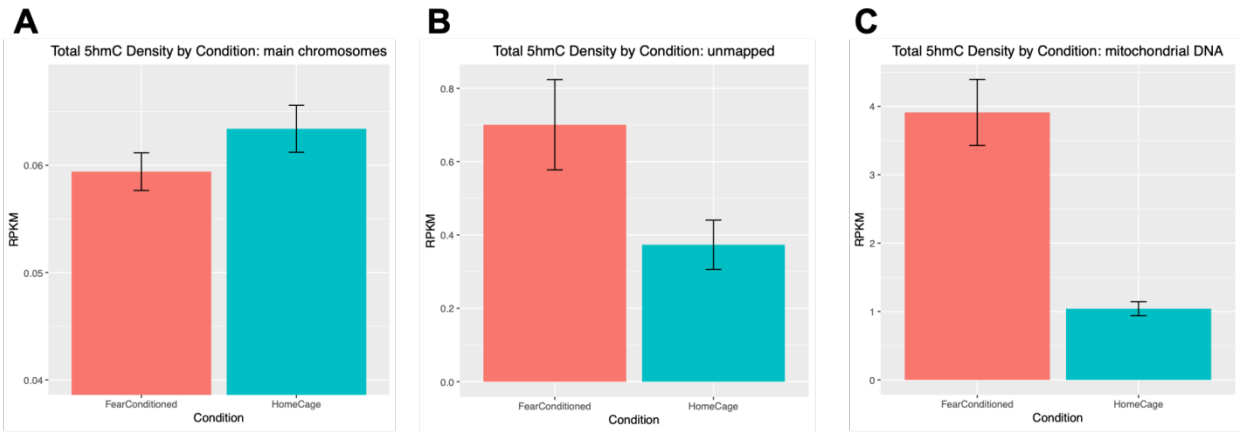
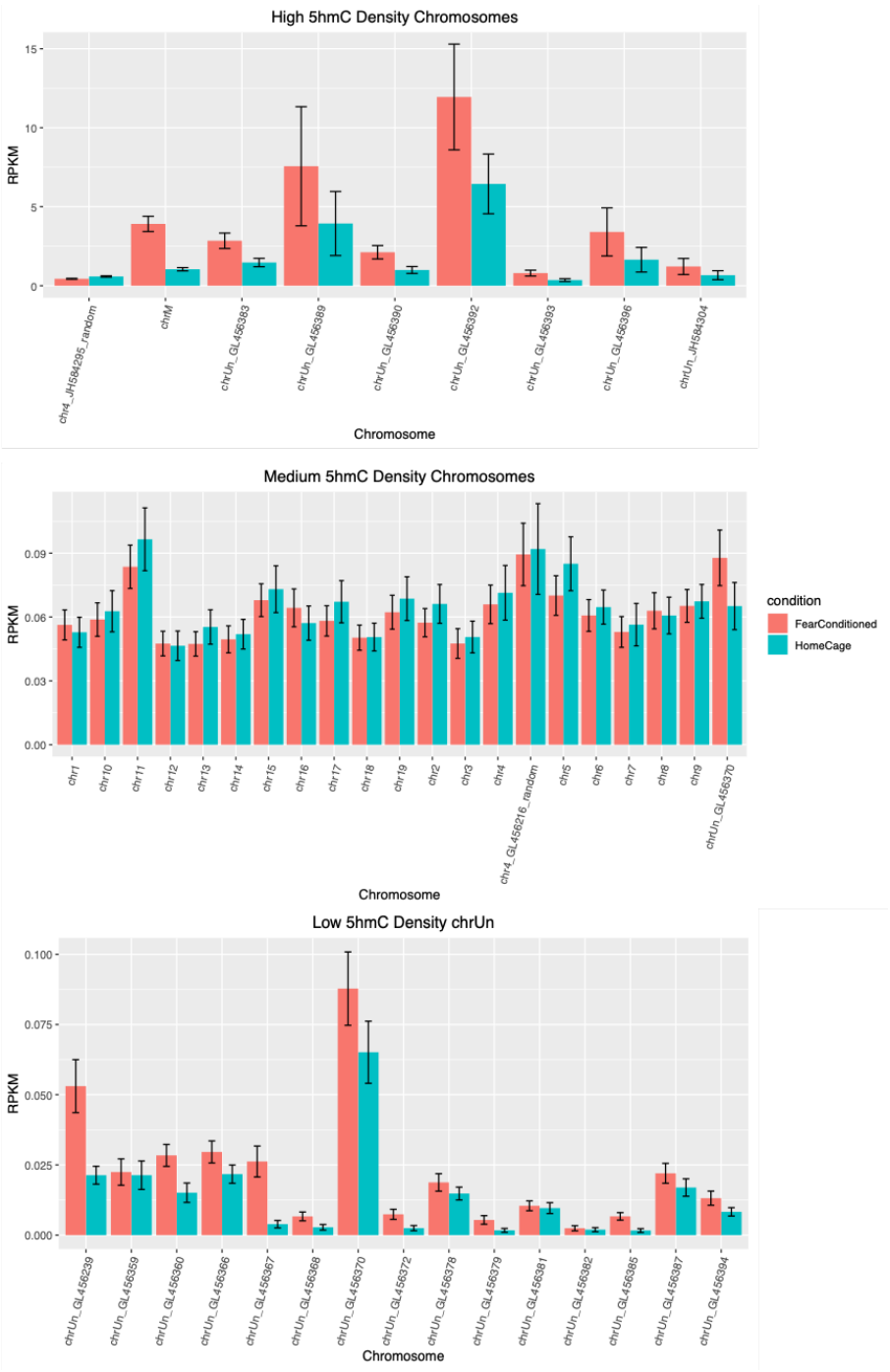


Figure 3. Genome-scale RPKM calculation of 5hmC by condition. (A) Total 5hmC density across all autosomes (chromosomes 1-21) and the sex chromosomes (chromosomes X and Y). (B) 5hmC density across unmapped chromosomal contigs. (C) 5hmC density across the mitochondrial genome. Error bars represent the standard error of the mean.



Supplementary Figure 4. Changes in 5hmC density between home cage and fear conditioning for each individual chromosome and partial contiguous regions. Error bars represent the standard error of the mean.

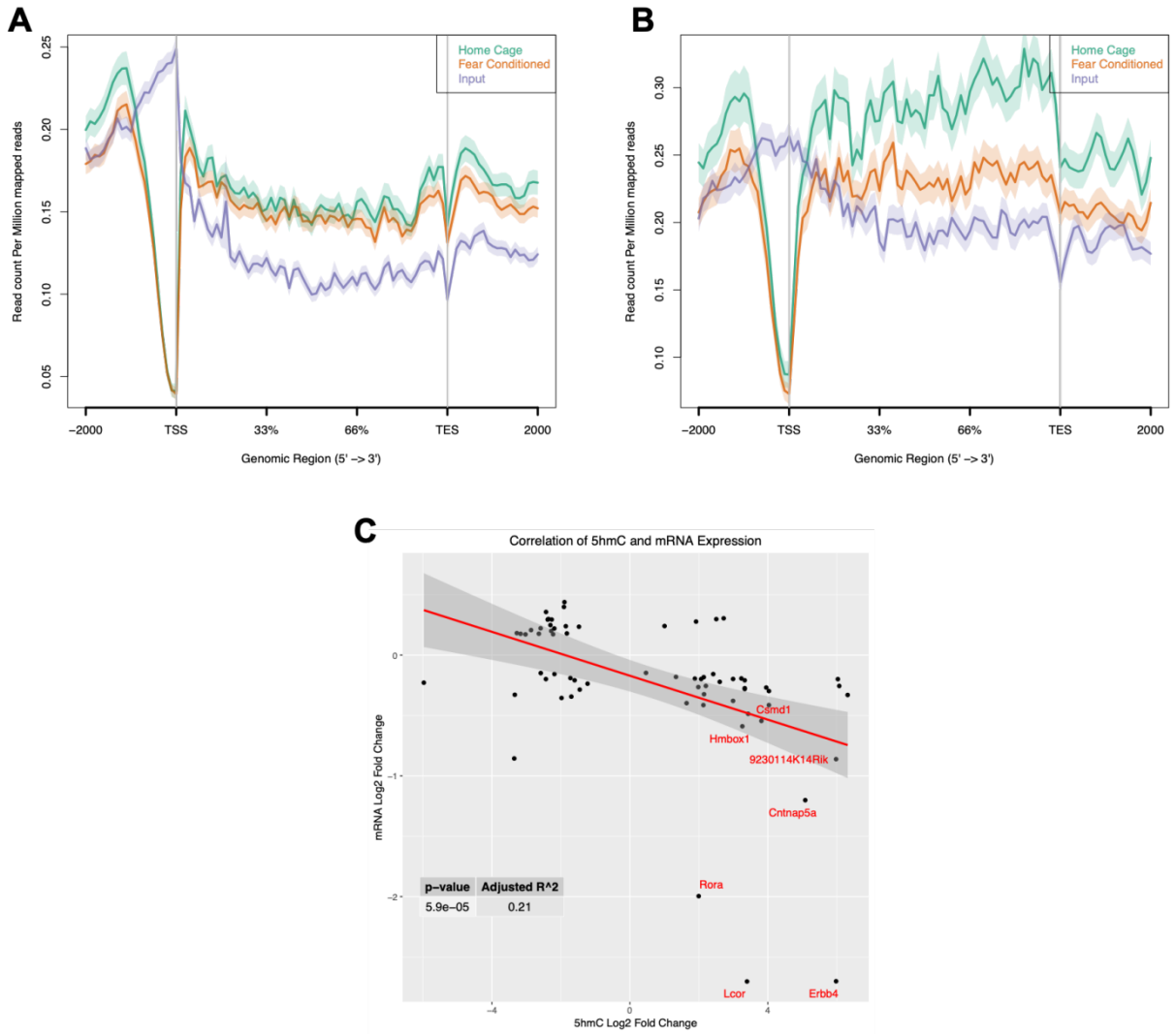
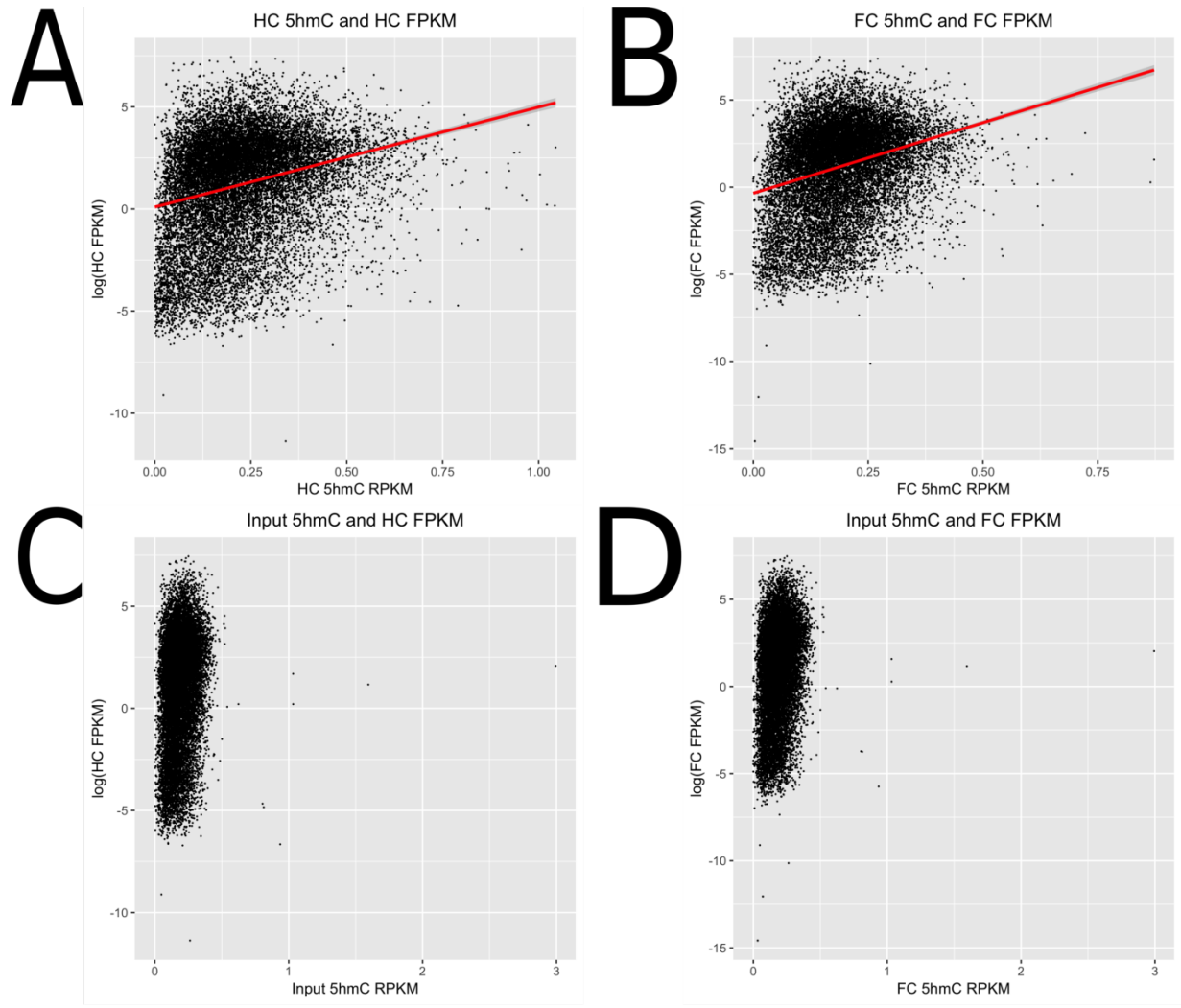


Figure 4. 5hmC density across gene bodies of all genes measures in by RNA-sequencing for (A) significantly downregulated transcripts and (B) significantly upregulated transcripts. (C) Correlation between log fold change of 5hmC in MEDIPS-DHMRs contained within gene bodies of significantly differentially regulated gene transcripts. MEDIPS DHMRs were called using a q-value cutoff of 0.3 and significantly differentially expressed genes were called using a q-value cutoff of 0.1. In all graphs shaded regions represent a probability density distribution.



Supplementary Figure 5. Correlation between gene body 5hmC and gene expression. (A) Home cage (p-value < 2 X 10^{-16} and adjusted pearson R^2=0.005088) and (B) fear conditioned (p-value < 2 X 10^{-16} and adjusted pearson R^2=0.005025) 5hmC density as RPKM on the x-axis plotted against raw expression values; averaged across all replicates for both measures. The correlation between home cage (C,D) Input 5hmC density across gene bodies compared to expression values in both conditions.

Pathways Regulated by Fear Conditioning

GO PATHWAY	ENRICHMENT SCORE: FC vs HC	FWER
NADH DEHYDROGENASE ACTIVITY	1.843	0.073
NADH DEHYDROGENASE COMPLEX	1.832	0.075
NADH DEHYDROGENASE COMPLEX ASSEMBLY	1.846	0.081
IRE1 MEDIATED UNFOLDED PROTEIN RESPONSE	1.814	0.087
NEGATIVE REGULATION OF PROTEIN KINASE B SIGNALING	1.779	0.095
RESPIRATORY CHAIN	1.795	0.096
MITOCHONDRIAL ELECTRON TRANSPORT NADH TO UBIQUINONE	1.790	0.096
CHAPERONE MEDIATED PROTEIN FOLDING	1.784	0.096
MITOCHONDRIAL RESPIRATORY CHAIN COMPLEX I ASSEMBLY	1.846	0.097
HSP90 PROTEIN BINDING	1.800	0.098
MYELIN SHEATH	1.947	0.104
INNER MITOCHONDRIAL MEMBRANE PROTEIN COMPLEX	1.872	0.106
RELEASE OF CYTOCHROME C FROM MITOCHONDRIA	1.903	0.106
MITOCHONDRIAL PROTEIN COMPLEX	1.761	0.113
MITOCHONDRIAL RESPIRATORY CHAIN COMPLEX I BIOGENESIS	1.846	0.121
HEAT SHOCK PROTEIN BINDING	1.731	0.128
ATP BIOSYNTHETIC PROCESS	1.734	0.131
OXIDATIVE PHOSPHORYLATION	1.736	0.135
PROTEIN FOLDING	1.721	0.137
UNFOLDED PROTEIN BINDING	1.736	0.143
NEGATIVE REGULATION OF TRANSCRIPTION REGULATORY REGION DNA BINDING	1.709	0.144
MITOCHONDRIAL RESPIRATORY CHAIN COMPLEX ASSEMBLY	1.711	0.147
TRANSLATION ELONGATION FACTOR ACTIVITY	1.699	0.155
MITOCHONDRIAL MEMBRANE PART	1.684	0.178
ENERGY COUPLED PROTON TRANSPORT DOWN ELECTROCHEMICAL GRADIENT	1.651	0.234
REGULATION OF ENDOPLASMIC RETICULUM UNFOLDED PROTEIN RESPONSE	1.642	0.242
ATP SYNTHESIS COUPLED PROTON TRANSPORT	1.651	0.243
ELECTRON TRANSPORT CHAIN	1.654	0.244
REGULATION OF CELLULAR RESPIRATION	1.644	0.245
REGULATION OF GENE EXPRESSION BY GENETIC IMPRINTING	-1.780	0.244

Table 1. Gene Set Enrichment Results (GSEA) for RNA-seq comparing home cage to fear conditioned animals.

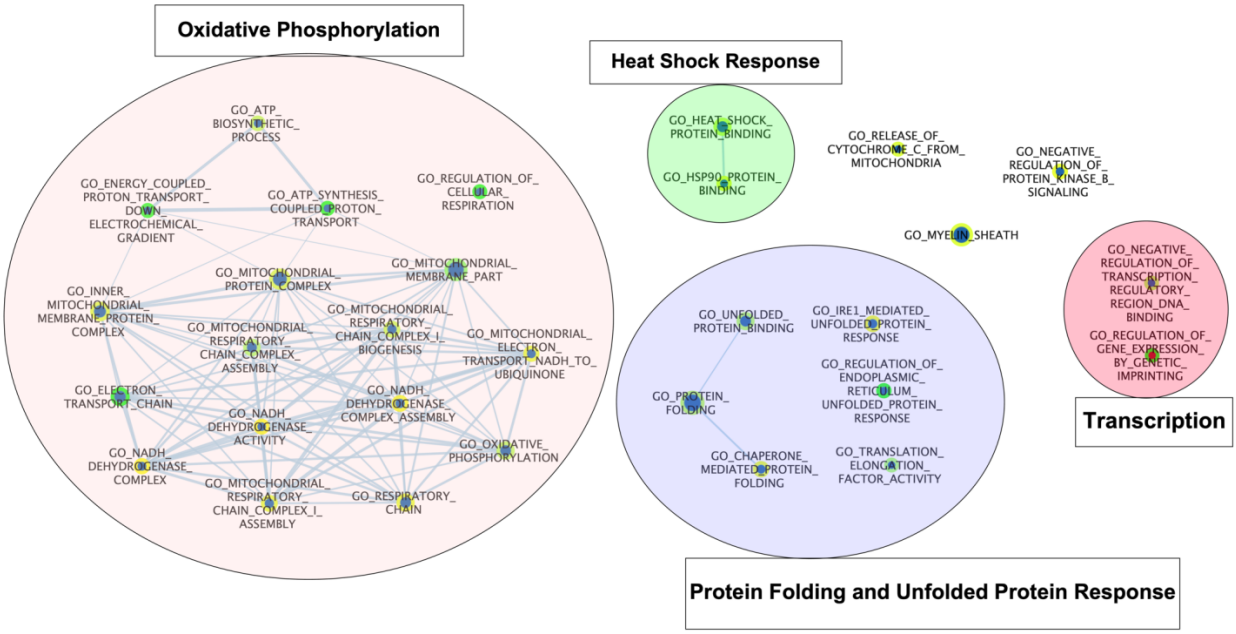
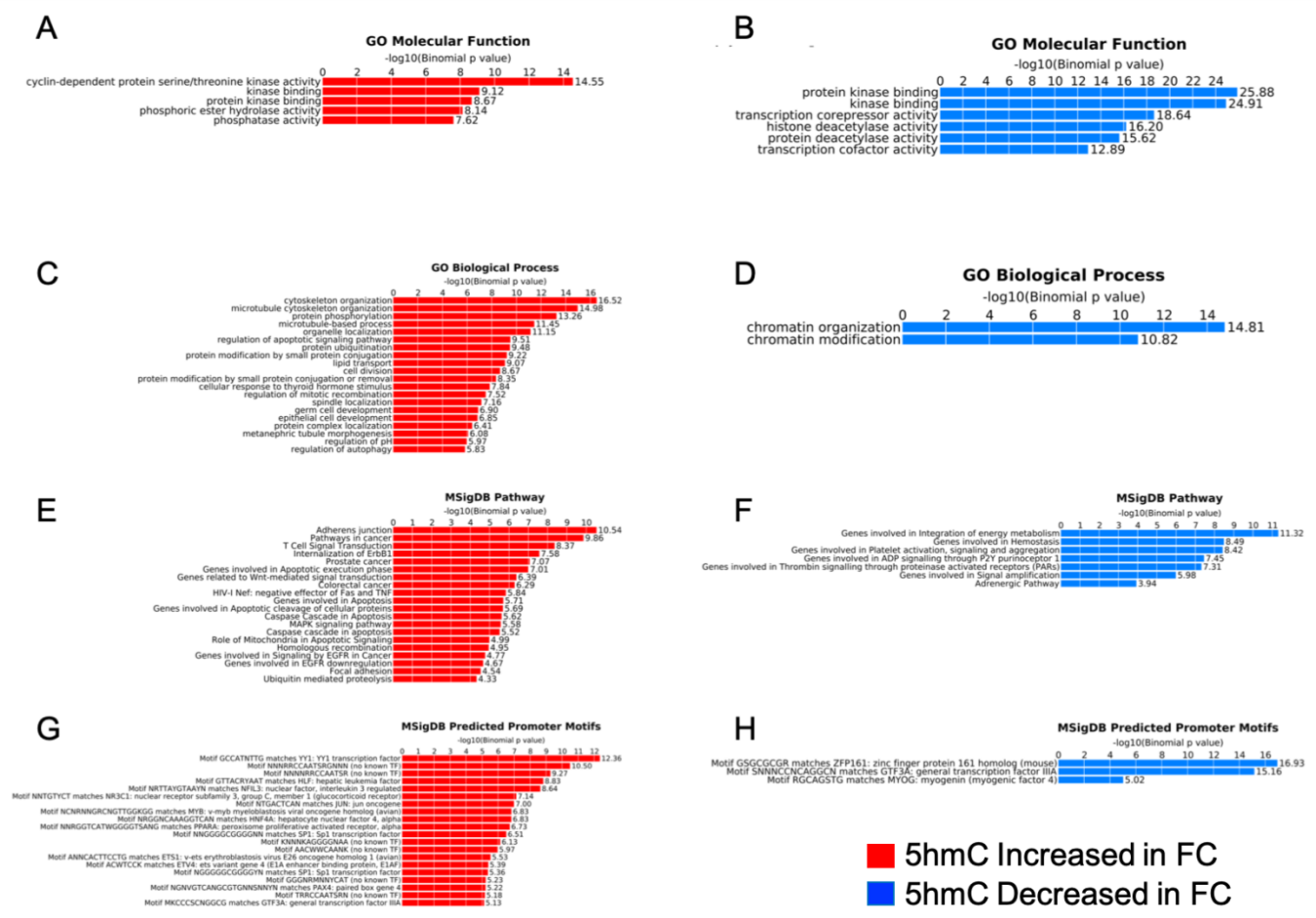


Figure 5. Network representation of pathways modulated by fear conditioning. Each point represents a pathway, with blue centers corresponding to pathways enriched in fear conditioning as compared to home cage and red centers visa versa. The border color is a continuous spectrum determined by the Q-value, with yellow corresponding to high significance and green corresponding to low significance values, as displayed in Table 1. The edges between pathways correspond to the number of genes shared between those sets, with thicker edges corresponding to a larger number of shared genes.



Supplementary Figure 6. Pathway analysis of 5hmC peaks by GREAT. GO Molecular Function pathways significantly associated with (A) MACS DHMRs with 5hmC increased in fear conditioning (FC) or (B) 5hmC reduced with fear conditioning; similarly for GO Biological Process (C,D), MSigDB Pathways (E,F), and MSigBD Predicted Promoter Motifs (G,H).

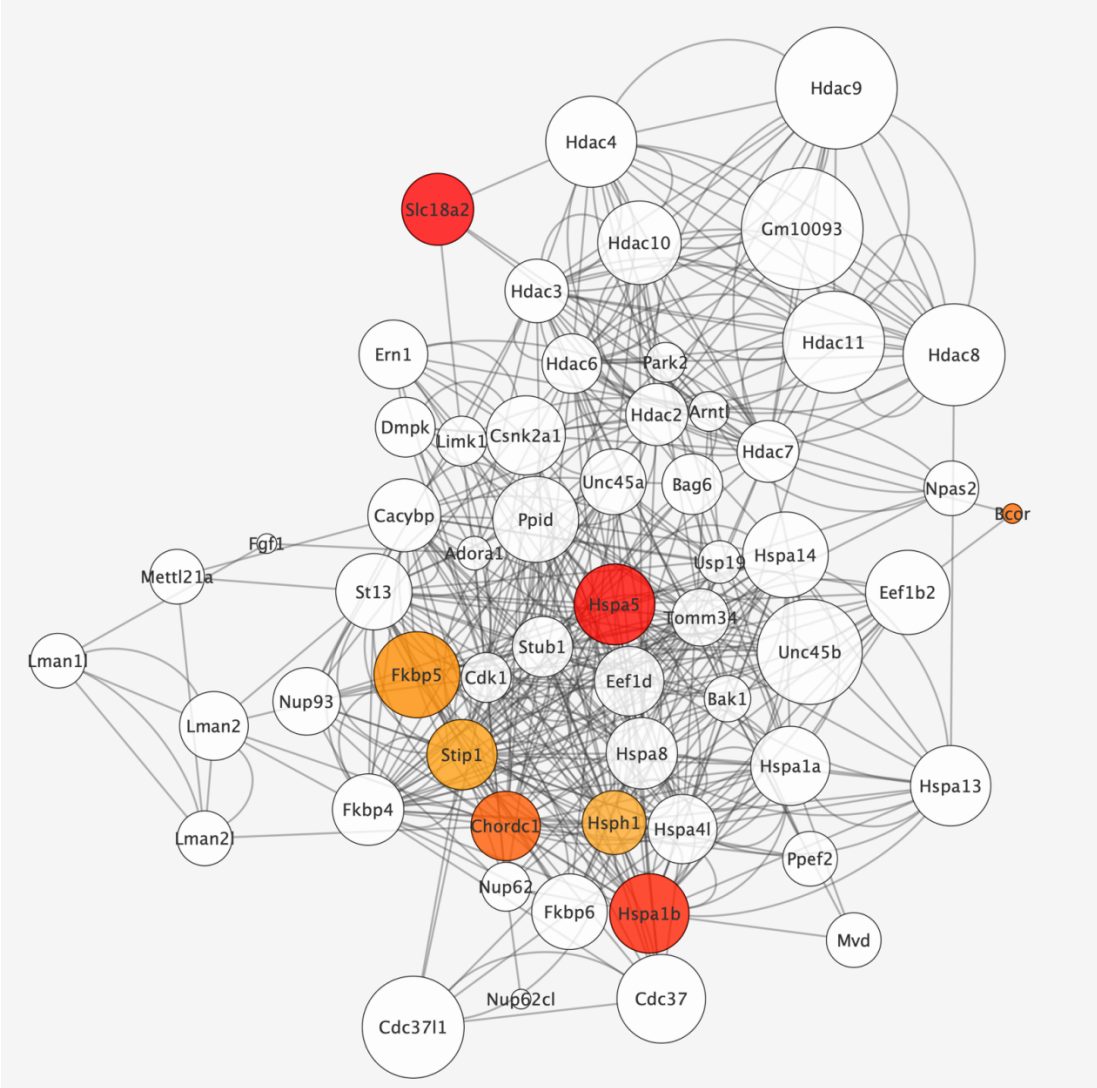


Figure 6. Gene network derived from the GO: Heat Shock Protein Binding set. Colored are the genes differentially regulated during fear conditioning with more redder nodes corresponding to more downregulated genes. Node size corresponds to the clustering coefficient, with larger size = greater coefficient. The local clustering coefficient is a measure of local density from graph theory, indicating to what extent a gene forms a network or acts as a local hub. (Barabási & Oltvai, 2004)

name	sequence
fgfr1_1_59.28_BSSeq_F	TTGTAGATTAGGTTGGTTTGGAATT
fgfr1_1_58.98_BSSeq_R	ATCCCAACACTCTAAAAACAAAAAC
fgfr1_2_57.49_BSSeq_F	GATAGGGTTTTTTTGTGTAGTTTTG
fgfr1_2_54.38_BSSeq_R	AATAATCAAAAATATAACTTTTTAAAATTT
fgfr1_2_58.28_BSSeq_R	TTTTTTGTGTAGTTTTGGTTGTTTT
fgfr1_4_55.97_BSSeq_F	TTGTTAAGTAGTGGAGGATAGAGG
fgfr1_4_54.92_BSSeq_R	CAACTCCAAAAATAAAAAAATTATC
fgfr1_4_59.55_BSSeq_F	TTTGGTTGTGGGTTATATTTGTTAAG
fgfr1_4_59.80_BSSeq_R	CAATCTCCTAATACCAACTCCAAAA
fgfr1_6_right_58.46_BSSeq_F	GGGTGTAGTTGGTGGAGAGTAAT
fgfr1_6_right_59.48_BSSeq_R	CTCCCTTTCAAACTAACAAAATTAAAA
fgfr1_6_left_58.70_BSSeq_F	TAGTTTAGTTTTGGGGAGTTTTTGT
fgfr1_6_left_57.36_BSSeq_R	CAACCAATTAATACTCTACACATCATC
fkbp5_1_right_57.48_BSSeq_F	GTTGGTTATGTTATAGTGTGGGATT
fkbp5_1_right_56.51_BSSeq_R	CCATAATATTTCTATAAAAACAAAAACC
fkbp5_1_left_52.17_BSSeq_F	AAATAAGAATAAATTTATAAGGAATTTA
fkbp5_1_left_52.63_BSSeq_R	AAACTAAAAAACTATAAACAACACC
fkbp5_2_57.39_BSSeq_F	GGGATTAATTTTTAGAATTAAAGAAAAA
fkbp5_2_left_57.43_BSSeq_R	AAAATCTACCTACCTTTACCTCCC
gabrb2_1_57.49_BSSeq_F	TTGTAAGAGTAAAGAAAAGGAAGTTAGTAA
gabrb2_1_58.76_BSSeq_R	CACCATAAACACAAAACAAATACATC

Table S1. Primers used for targeted bisulfite sequencing.

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Chapter 3: Behavioral model of a Stress x Fear Interaction

Introduction

The diathesis-stress hypothesis of psychiatric disease, first put forward in the 1800's, posits that the interaction between life stressors and genetic predisposition are the root cause of the emergence of many psychiatric disorders, including PTSD (Post-traumatic Stress Disorder). (Ingram & Luxton, 2005; McKeever & Huff, 2003) Studies of PTSD have demonstrated that a subset of individuals who experience trauma go on to develop PTSD psychopathology – which includes aberrations in fear learning. (Briscione, Jovanovic, & Norrholm, 2014) Fear learning is an adaptive process that is crucial to organismal survival. Stressful experiences engage with the neural circuitry of learned fear to increase recognition of a potentially dangerous environment. A hallmark of PTSD is that this process becomes over-active. To be better understand how stress interacts with neurophysiology, animal models of disease have been key entry points.

Animal models of stress have revealed many of the adaptive responses to stress, both acute and chronic. Stress has many somatic effects, as the psychosocial stress response is fundamental to driving important physiological adaptations to better face the environment. Understanding the normal physiological responses to stress can help us to better elucidate pathways that may be sensitized by genetic predisposition or interacting life experiences leading to maladaptation and disease pathology. Stress causes neurophysiological changes ranging from gross alterations in brain structure and size, down to synaptic alterations. Stress results in hippocampal size reduction– an effect seen both in rodent models of stress and in numerous human disease states. (Frodl et al., 2006; Rahman, Callaghan, Kerskens, Chattarji, & O'Mara, 2016) Functional connectivity between neural circuits is also modulated with stress. In particular, stress results in amygdala to hippocampal circuits with increased functional connectivity while

intra-hippocampal (regions CA1 to CA3) regions demonstrate reduced connectivity. These data suggest an increased amygdalar dominance over neural processing (Ghosh, Laxmi, & Chattarji, 2013) Neuronal complexity is also increased as a result of stress, and interestingly, stress appears to have diverse consequences on neuronal structure depending on which brain region one is querying. (Chattarji, Tomar, Suvrathan, Ghosh, & Rahman, 2015; Vyas, Jadhav, & Chattarji, 2006) In addition synaptic electrophysiological alterations, such as increased excitatory neuronal LTP in the BLA and concomitant reduction in LTP in the hippocampus, are seen with chronic stress (Narayanan & Chattarji, 2010; Suvrathan et al., 2014).

Zooming in to the function of molecular circuits within neurons, there are also major shifts in the transcriptional profiles of central nervous system tissues associated with stress. Certain molecular pathways appear to be important to mediate stress effects in the BLA, furthermore some of these appear to have tissue specificity. In particular, studies manipulating the expression of genes prior to the induction of stress have shown that particular pathways are crucial for the molecular adaptations that occur. Fatty acid amid hydrolase (FAAH) is necessary for the increased BLA dendritic arborization, complexity and spine density of pyramidal neurons seen with stress; and the attendant anxiogenic behavioral outcomes of stress. (Hill et al., 2013) Another interesting factor is tissue-plasminogen activator (TPA), which is necessary for stress-induced morphological changes in the medial amygdala, but not BLA. (Bennur et al., 2007) These important studies demonstrate that the anatomical consequences of stress are dependent on particular molecular pathways. A landmark investigation of early life stress demonstrated that transient, juvenile, downregulation of the transcription factor *Otx2* confers susceptibility to adult social defeat, whereas its over-expression during adolescence, can rescue social interaction

deficits conferred by early life stress. (Peña et al., 2017) This study pointed out how a transient epigenomic signature can set up a circuit for a stress-sensitized behavioral response later in life.

Stress can have pleiotropic effects on behavior. Some of the most reliable readouts of stress history in rodent models have been motion-based assays of anxiety-like behavior, with all of the attendant pitfalls of animal models of disease. However, as others in the field have argued, the objective study of observable behaviors across species can offer valuable insight into "emotional primitives" that are demonstrable across species and reflect the engagement of homologous or orthologous neural circuitry driving these shared responses. (Anderson & Adolphs, 2014) Fear and anxiety-like outcomes are particularly suited to this sort of analysis, as the observable behavioral outcomes are similar and indeed the brain regions involved in the expression of such behavior are shared between mouse and man. The field of behavioral neuroscience has developed and validated a variety of assays to assess fear- and anxiety-related types of behavior, and studies have demonstrated causal associations between neural and molecular circuitry and these phenotypes. (Asok, Kandel, & Rayman, 2018; Bourin, 2015; Bowers & Ressler, 2015; Calhoon & Tye, 2015; Duvarci & Pare, 2014; Giustino & Maren, 2015; Lezak, Missig, & Carlezon Jr, 2017)

The NIMH's Research Domain Criteria (RDoC) have been established specifically to encourage the development of a frame work that: (1) defines the domains of biology that are relevant to psychiatric illness, (2) clarifies the variation in these domains from normal to abnormal, (3) develops useful measures for these domains, and (4) integrates multiple levels of scientific inquiry to understand these domains and emergent disease. (Cuthbert & Insel, 2013) The application of this useful RDoC framework to PTSD has pointed towards negative valence systems (increased fear, anxiety, and avoidance), positive valence systems (reduced interest,

appetite, libido, dysregulated reward and motivational behavior) and increased sympathetic nervous system activity (nervousness, sleeping problems, startle response , etc). (Schmidt & Vermetten, 2018) Focusing on negative valence systems, neurophysiological studies of PTSD patients have demonstrated specific correlates of abnormal fear processing, including enhanced fear memory expression, deficits in fear inhibition, increased generalization of fear, and deficits in fear extinction. (Briscione et al., 2014; Jovanovic et al., 2005, 2009, 2010; Norrholm et al., 2011) Herein we sought to establish a model of traumatic experience with the capacity to recapitulate a subset of these negative valence behaviors observed in C57BL/6 mice. Furthermore, we worked to validate a paradigm that works reliably in the hands of both male and female scientists. Finally, we validated alterations in key stress pathway genes in the brain.

We sought to establish and validate a model of stress-induced fear memory to assess the acute effects of stress on fear parameters. Many stress paradigms in the field demonstrate inconsistent results across experimenters and facilities, and it is not understood exactly what underlies these differences. Furthermore, there is a paucity of well-defined and accessible paradigms to understand the interaction between stress and fear-consolidation, an important and distinct component of fear learning that is dysregulated in patients with PTSD. Paradigms have been established that reflect deficits in fear extinction. (Andero, Dias, & Ressler, 2014; Andero et al., 2013; Long & Fanselow, 2012; Maren & Holmes, 2016; Miracle, Brace, Huyck, Singler, & Wellman, 2006) And paradigms have been established that demonstrate enhanced sensitization to contextual fear conditioning, contingent upon the delivery of unsignaled footshocks. (Rajbhandari, Gonzalez, & Fanselow, 2018; Rau & Fanselow, 2009) This is a robust and long-lasting behavioral effect, but is hampered by the use of a shock as both the stressor and the US, and is also limited by the use of contextual conditioning only. Here we adapt and extend

a paradigm originally presented in Baratta et al., 2016 that uses restraint stress to sensitize auditory fear conditioning, resulting in enhanced fear memory consolidation. (Baratta et al., 2016) We modify the stressor and fear conditioning parameters, demonstrate increased fear expression to the trained auditory cue, increased generalization to tones of similar frequencies, and increased anxiety-like behavior that exhibits a dose-dependent response to stress. We also demonstrate that in terms of fear conditioning, this stress specifically influences fear expression and does not influence fear extinction, reinstatement, or spontaneous recovery. And finally, we demonstrate that this paradigm is robust in the hands of multiple experimenters, both female and male. As an added benefit, this paradigm has a quick turnaround, with a low hands-on requirement, and can be highly parallelized to include many animals in each cohort – a particularly important requirement when the full extent of individual behavioral variation is to be captured.

Results

Previous work has demonstrated that stress can dose-dependently influence corticosteroid response, neural response, and the behavioral sequalae of stress. (Melia, Ryabinin, Schroeder, Bloom, & Wilson, 1994; Natelson et al., 1988; Pitman, Ottenweller, & Natelson, 1988) Chronic stress paradigms or long periods post-stress for adaptation can fundamentally alter neural architecture. (Chattarji et al., 2015) We aimed to unravel the short-term interaction of stress and fear- and anxiety-related circuity – before extensive circuit remodeling occurs and contributes to behavior. We compared the acute anxiogenic effects of 1 or 3 consecutive days of restraint stress. Briefly, we restrained mice for either 3 consecutive days, or 1 day, and 30 minutes after the completion of restraint (or 3 days of control handling) we introduced the mice to an open

field to assess the acute anxiogenic effects of restraint. Twenty four hours later, mice were fear conditioned, and twenty four hours after that mice were tested for recall of the conditioned fear memory. (methods) (Figure 1)

Immobilization-stress results in a non-normal distribution of anxiety-like behavior

We first sought to understand the distribution of values for stress-sensitive readouts in the open field. Work from others has suggested that the behavioral effects of stress may exhibit a skewed distribution. (Anthony et al., 2014) This distribution of behavior may be a result of differences in individual response. These large individual differences in stress response are reflected at the biological level as well – with stress sensitive animals shown to have greater expression of stress related genes, and increased electrophysiological correlates of stress in relevant brain regions. (Jakovcevski, Schachner, & Morellini, 2008; Meyer, Burgos-Robles, Liu, Correia, & Goosens, 2014; Mozhui et al., 2010) To this end, we analyzed the distribution of behavioral readouts in the open field by group. We found that the ratio of time spent in the periphery to time spent in the middle of the open field demonstrated significant deviations from the normal distribution. (Shapiro-Wilks test of normality p-value = 0.015) Whereas control measures (i.e. measures that are not thought to be sensitive to anxiety-like behavior), such as time spent in the middle zone of the open field, demonstrated a normal distribution. (Shapiro-Wilks test of normality p-value = 0.95) (Figure 2)

Immobilization stress increases anxiety-like behavior

To measure the main effect of immobilization stress on anxiety, we assayed the ratio of the time spent in the periphery by the time spent in the center (peri/center ratio), and the related

measure of the percent of time spent in the center of the open field. We found that mice immobilized for 3 consecutive days demonstrated both a greater peri/center ratio (Wilcoxon p-value = 0.018) as well as a reduced percentage of time spent in the center of the open field (Wilcoxon p-value = 0.029).

We next asked whether the dose of stress delivered would impact the behavioral readout of stress. We found that a single session of stress trended towards an increased ratio of the time spent in the periphery as compared to the center of the open field (Wilcoxon p-value = 0.011), as compared to controls. And three consecutive days of stress further increased this ratio from control (Wilcoxon p-value = 0.08). (Figure 4) These data suggest that this readout of anxiety-like behavior after immobilization stress is sensitive to the dose of the stressor used.

Immobilization stress potentiates fear response

After the final immobilization session, the next day mice underwent auditory fear conditioned (methods) using a 50% CS-US pairing paradigm. A previous study demonstrated that this paradigm was sensitive to the effects of stress, and it also may be that the effects of stress may enhance the learning of ambiguous cues. (Baratta et al., 2016; Tsetsenis, Ma, Iacono, Beck, & Gross, 2007) The 3X-IMMO mice were compared to control mice that were handled during the time when experimental mice were immobilized. Both groups acquired fear at the same rate, as measured by within-session freezing to tones. (Figure 5a) This suggested that any resulting differences in fear memory are not attributable to increased learning of fear. Twenty-four hours after fear conditioning, mice were subject to fear expression testing. (methods) Both groups demonstrated very low pre-CS freezing, indicative of low basal anxiety to the context and appropriate contextual shifting. Freezing during the tone was significantly increased in the

immobilized group of animals as compared to the control group. (Figure 5b) (Student's t-test, p = 0.028)

A History of Immobilization Stress Potentiates Fkbp5 Expression in the Central Amygdala

In order to further validate our model, we sought to identify molecular markers of stress that track a history of immobilization stress. To do so, we profiled the expression of the *Fkbp5* gene in key brain regions in control fear conditioned mice, as compared to immobilized-fear conditioned mice. *Fkbp5* is a key mediator of the HPA axis, is implicated in brain glucocorticoid receptor sensitivity, and stress mediated signaling in rodent and primates, and is widely associated with human disease. (Hartmann et al., 2012; Scammell, Denny, Valentine, & Smith, 2001; Scharf, Liebl, Binder, Schmidt, & Muller, 2011; Touma et al., 2011) Not only does *FKBP5* regulate glucocorticoid receptor signaling, it is itself regulated by glucocorticoid receptor signaling, being transcriptionally activated by glucocorticoid receptor binding in promoter and regulatory regions. (Hubler et al., 2003; Hubler & Scammell, 2004; Magee, Chang, Stormo, & Milbrandt, 2006) Thus it is proposed that *FKBP5* acts as an ultra-short negative feedback loop intracellularly, to oppose the signaling pathways of particular major hormones, with the glucocorticoid pathway being of particular relevance to PTSD. Given this role of *Fkbp5* in regulating the HPA axis, and in some sense recording the history of stress at a molecular and epigenetic level, we reasoned that *Fkbp5* expression dynamics in key brain regions should be differentially modulated during the fear memory consolidation process by immobilization stress.

We profiled *Fkbp5* in 4 brain regions where *Fkbp5* has been shown to be differentially modulated by stress: the basolateral amygdala (BLA), the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (Bnst) and the periventricular nucleus of the

hypothalamus (Pvn). (Scharf et al., 2011) We found that a history of stress potentiated *Fkbp5* expression specifically in the CeA. (ANOVA p-value = 0.02, tukey post hoc p-value = 0.019). The CeA is one of the most robustly stress regulated Fkbp5 brain regions, and also is intimately involved in computing the response to auditory fear conditioning. (Li et al., 2013)

Discussion

The complexity and multifactorial nature of complex psychiatric disorders has necessitated the development of targeted approaches to understanding the neural circuitry dysregulated by disease. In PTSD, the dysregulation of negative valence systems, has been well-studied in human populations. In particular, aberrations in specific facets of fear learning have been well-established in PTSD patients, including enhanced fear learning or fear load, increased generalization of learned cues, reduced extinction of fear-associated cues, and reduced discrimination of safe and unsafe cues. (Briscione et al., 2014) Herein, we present a model of restraint-stress enhanced fear learning, that is robust, reproducible by multiple experimenters, and scalable. Our model demonstrates increased anxiety-like behavior and enhanced fear expression in animals with a history of stress. We also demonstrate a stress-dependent potentiation of *Fkbp5* expression in the central amygdala.

Sources of variation in behavioral neuroscience are difficult to address, unincentivized by funding sources or publishers, and likely drive the reproducibility issues in studies utilizing behavioral readouts as the end-point. (Bohlen et al., 2014; Chesler, Wilson, Lariviere, Rodriguez-Zas, & Mogil, 2002) One large study posited that idiosyncratic results across laboratories are highly likely, while others have suggested that certain behavioral assays of state traits may be more reproducible. (Crabbe, Wahlsten, & Dudek, 1999; Mandillo et al., 2008)

Furthermore, experimenter variation in terms of protocol experimenter experience, and gender may also play important roles in the response of rodents to behavioral testing – particularly in terms of anxiety-like behavior. (Hurst & West, 2010; Sorge et al., 2014; van Driel & Talling, 2005) However, rodent behavioral assays offer a crucial, and valuable insight into the biological substrates underpinning complex phenotypes. Mouse models of stress-induced dysfunction have largely focused on anxiety , however, fewer models exist at the intersection of stress and learned fear in mice.

Herein, we present a model that recapitulates the influence of stress on fear memory consolidation circuitry, that may be seen in PTSD patients. The quick turnaround time and scalability of this behavioral paradigm can allow a single experimenter to process a large number of mice, allowing for one to capture a fuller spectrum of variability. This model offers a starting point to study the effects of a short-term stressor on learned fear.

Methods

Immobilization Stress

Mice were immobilized for 1 hour in decapicone plastic bags (Braintree Scientific). The decapicone cone-shaped bags, were held open and the mouse was introduced into the wide opening. The mouse was secured in the bag, using electrical tape to close the wide opening, while ensuring the bag was not too tight to inhibit respiration and the snout was protruding from the opening at the small end. Bagged mice were then place in an empty plastic box in a supine position and were monitored for the duration of the immobilization procedure. After 1 hour of immobilization, the electric tape was released, and the mouse was removed from the bag by its tail and placed back into its cage.

Open Field Testing

Mice were placed in the corner of an open field (44 × 44 × 30 cm) and allowed to explore freely for 10 min while being video recorded. Light conditions in the open field were tuned to low light conditions, with the center of the field being ~90 lux and the edges of the field ~75 lux. Characteristics of locomotor activity were analyzed using Ethovision-XT (Noldus Information Technology). In this 3X-IMMO paradigm, mice were introduced to the open field 30 minutes after immobilization, in between which they were returned to their home cage. Control mice were introduced to the open field 30 minutes after handling, and were also returned to their home cage in between the two sessions.

Fear Conditioning and Expression

Fear conditioning and expression testing were carried out as previously described. (Baratta et al., 2016) Briefly, before fear conditioning, mice were removed from the animal care facility and put into a holding room proximal to the conditioning rooms for 1 hour. The holding room and conditioning rooms were distinct from the room used for immobilization. Standard rodent modular test chambers were used for conditioning ENV-008-VP; Med Associates Inc., St. Albans, VT) with an inside area of 30.5 cm (L) × 24.1 cm (W) × 21.0 cm (H). For fear conditioning, mice were conditioned to using a 50% CS-US pairing protocol. The CS was a 30 second, 2200 kHz tone at 80 dB; the US was a 0.5 second, 0.6 mA shock delivered coterminally with two of the tones, or delivered in the inter-tone interval for two of the shocks. There was a 180 second pre-CS period. The chambers were cleaned with quatricide in between mice, and the

room was illuminated with fluorescent ceiling lights. Mice were monitored with an overhead camera.

Before fear expression testing, mice were removed from the animal care facility and put into a holding room proximal to the conditioning rooms for 1 hour. Identical test chambers were used as for conditioning. However, the chambers were cleaned with 70% ethanol in between mice, 25 W red lights were used to light the boxes and the room, and black plastic slats were placed on top of the metal grid that prevented visual or physical contact with the grid floor. Mice were given a 180 second pre-CS period, after which a 180 second tone was played (2200 kHz at 80 dB). Mice were monitored with an overhead camera.

For both fear conditioning and expression video files from the overhead camera were analyzed using the FreezeFrame 4 video system. (Actimetrics)

Brain Dissection

For the analysis of *Fkbp5* expression, all mice were sacrificed 2 hours after fear conditioning either under control handling or 3X-IMMO conditions with brief isofluorane exposure and decapitation. Brains were immediately frozen on dry ice and stored at -80ºC. Samples were subsequently sliced on a microtome (25 micrometer) (Leica Biosystems SM 2000R) and 1 mm micro punches centered over the basolateral amygdala (BlA), central amygdala (CeA), bed nucleus of the stria terminalis (Bnst), and paraventricular nucleus of the hypothalamus (Pvn), were taken. With the exception of the Pvn, for which one central punch was taken, one brain punch per hemisphere was taken, alternating hemispheres. Shallow punches/scoops < 0.5 mm in depth were taken (as measured by careful slicing and optimization). Approximate coordinates, relative to bregma, for each brain region are below, according to the

Allen Mouse Brain Atlas (version 1, 2008). (Lein et al., 2007) Coordinates were matched as closely as possible to the highly differentially expressed loci for *Fkbp5* reported in Scharf, et. al, 2011. (Scharf et al., 2011) Brain punches were stored at -80°C until further processing.

Brain Region	**Medial-lateral (mm)**	**Anterior-posterior (mm)**	**Dorsal-ventral (mm)**
BLA	+/- 3.0	-1.7	-4.5
CeA	+/- 2.7	-1.00	-5
Bnst	+/- 1.00	-0.245	-4.5
Pvn	0	-0.755	-5.25

RNA Isolation, Reverse transcription, and qPCR

RNA was isolated by mechanical homogenization with a glass dounce (Wheaton cat: 357538) of the frozen brain punches in 0.5 mL Trizol reagent (Invitrogen cat: 15596026). RNA was extracted according to manufacturer's protocol. Briefly, trizol homogenized brain punches were extracted with a phenol:chloroform:isoamyl alcohol (25:24:1) solution, precipitated with isopropanol, sodium acetate, and glycoblue, air dried after ethanol washes, and then resuspended in molecular biology grade water (Corning cat: 46-000-CI).

RNA was quantified using a Qubit 2.0 Fluorometer using the RNA HS assay (Life Technologies cat: Q32852). 200 ng of RNA were reverse transcribed using the SuperScript IV First-Strand Synthesis System (Thermo cat: 18091200). Resulting cDNA was quantified by qPCR (Thermo: ViiA™ 7 Real-Time PCR System with 384-Well Block; Cat: 4453536). Briefly, 8 ng RNA equivalent of cDNA was used per reaction to quantify *Fkbp5*, normalized to *Ywhab*. (Primers: Fkbp5_forward: GGTTATCAAAGCCTGGGACAT;

Fkbp5_reverse: GAGCCATAAGCATATTCTGGTTTAC; Ywhab_forward: GGACACGAACTCTCCAATGAA; Ywhab_reverse: TTCTGTTCGATGCTGGAGATG) Ywhab was identified from re-analysis of genome-wide sequencing data from our group to identify invariant genes to be used as reference genes in the brain. Data published in Lori, et al., 2019. (Lori et al., 2018)

Figures

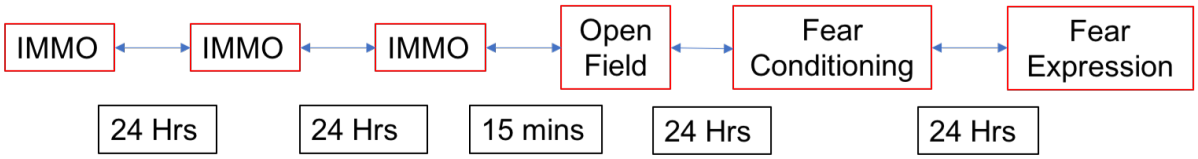

Figure 1. Schematic representation of the immobilization stress timing and testing of anxiety and fear behavior.

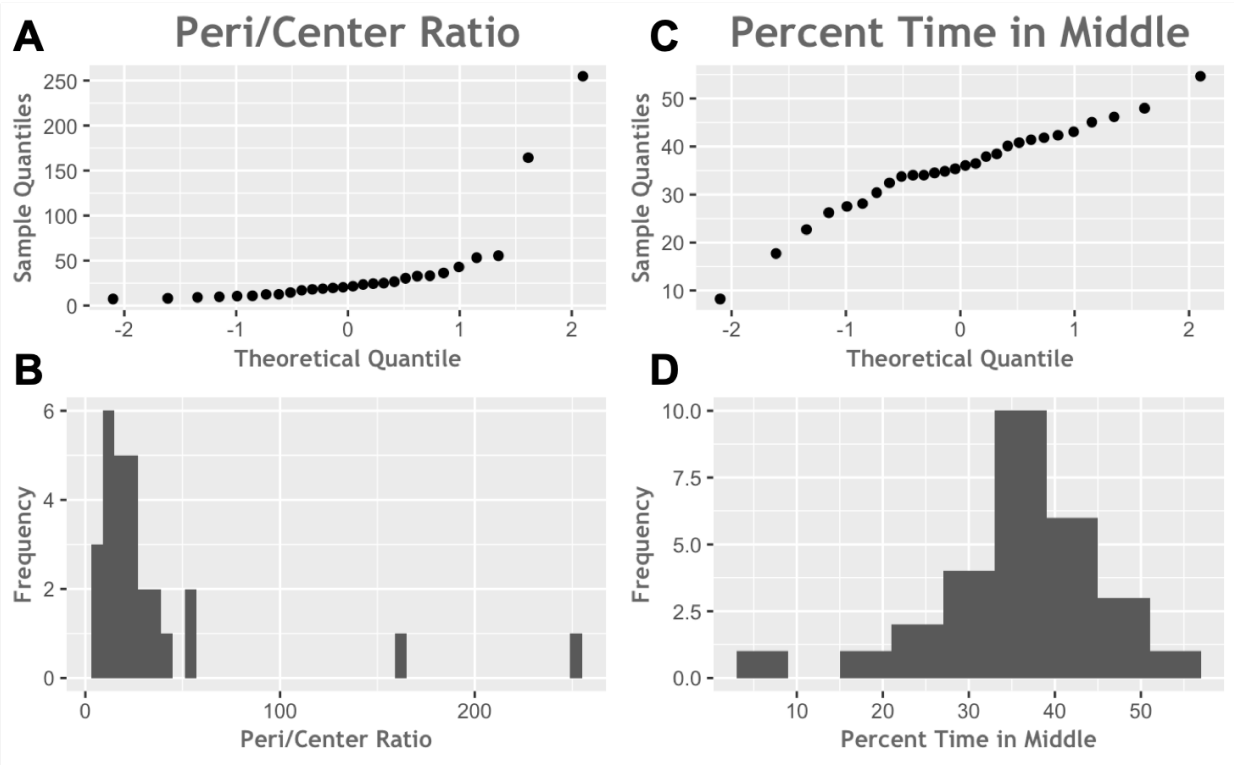

Figure 2. An analysis of normality across subsets of data obtained from open field experimentation. (a) QQ Plot of the ratio of percent time spent in the periphery and percent time spent in center; (b) histogram of the data in panel a; (c) a QQ plot of the time spent in the middle zone of the open field and (d) a histogram of panel c.

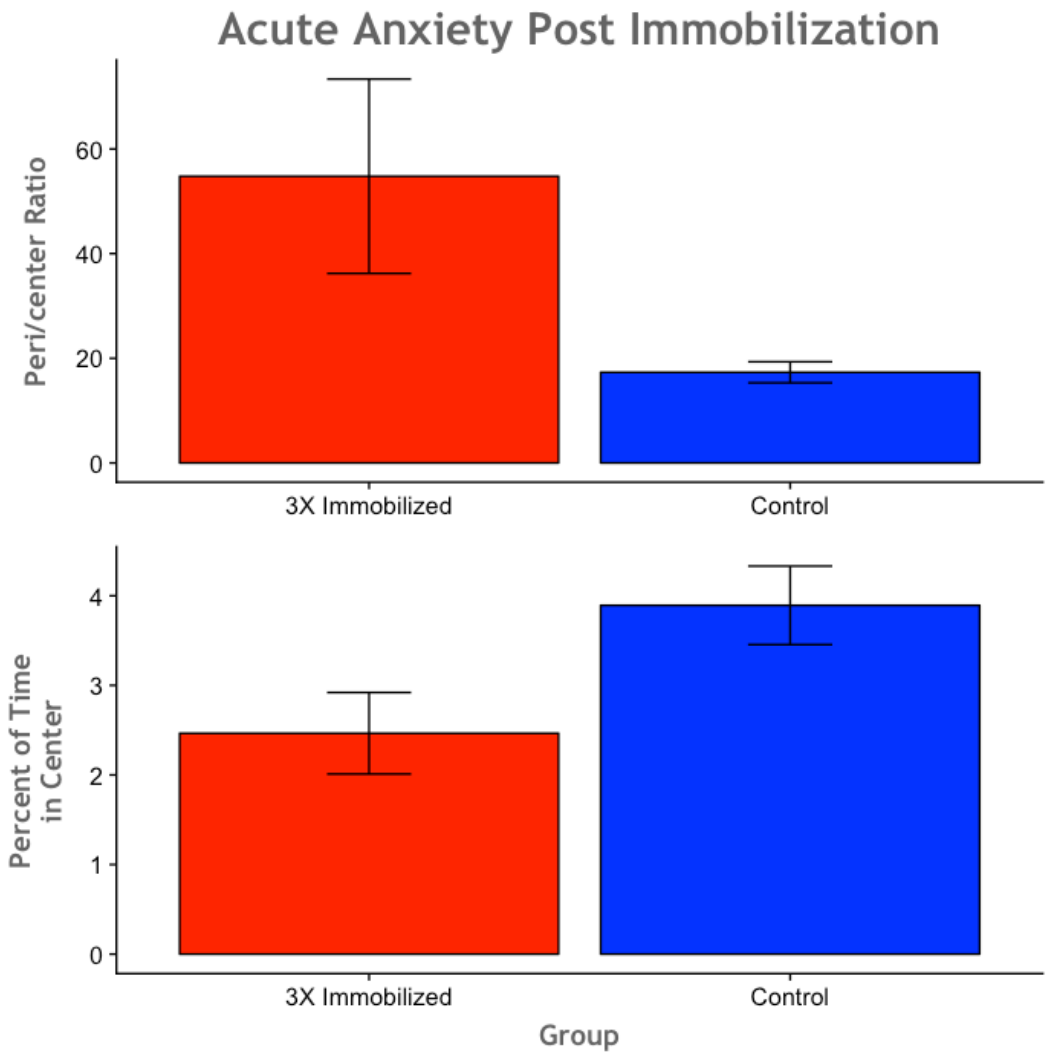

Figure 3. Replication of immobilization stress effect with (a) a comparison of the ratio of percent time spent in periphery to percent time spent in center; and (b) percent time spent in center. Error bars represent the standard error of the mean.

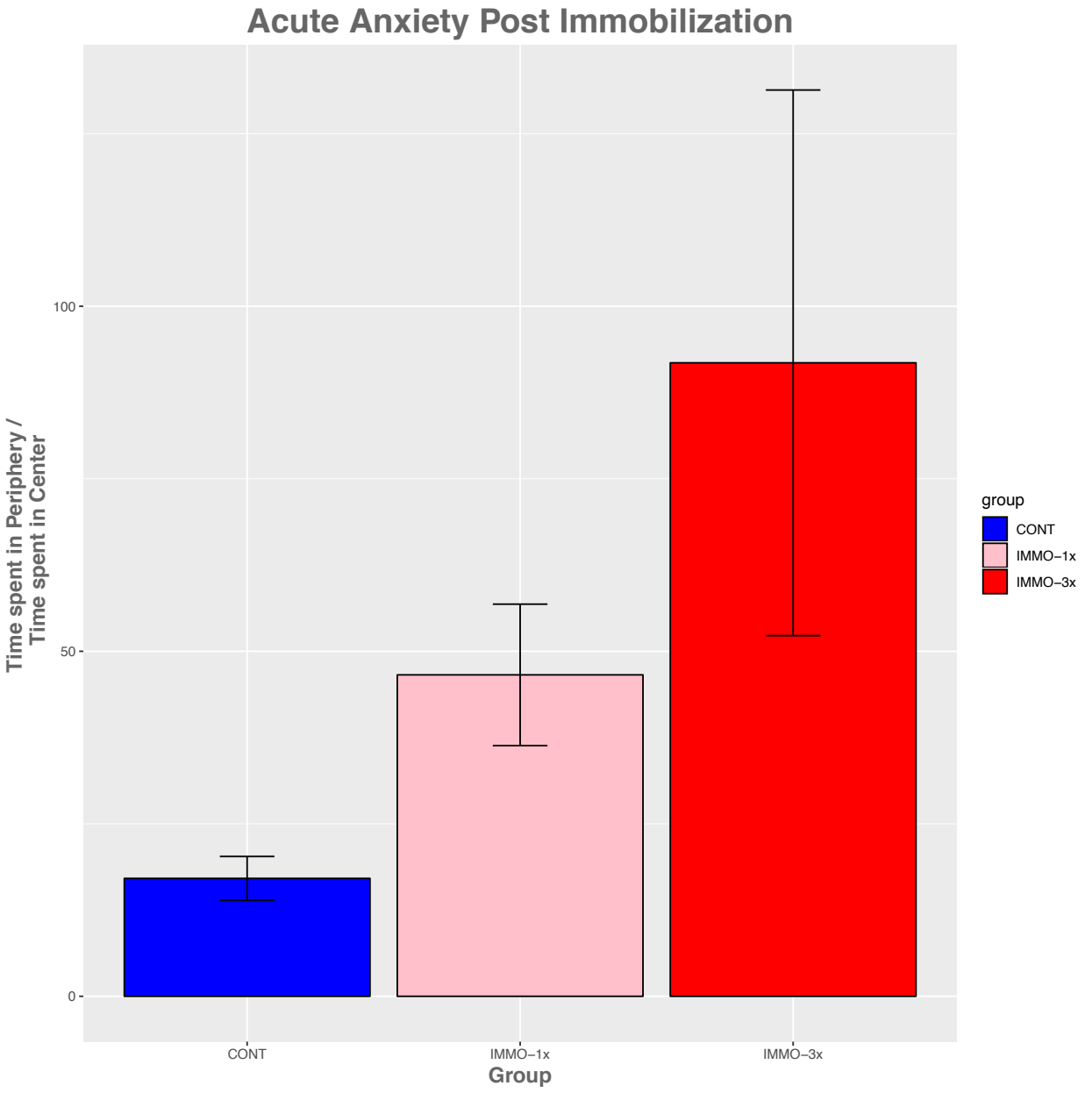

Figure 4. Stress dose response curve of open field behavior. Control mice were handled for 3 days, IMMO-1x mice were handled for 2 days and immobilized on day 3, and IMMO-3x mice were immobilized for 3 days. Mice were subject to open field analysis 30 minutes after either handling (control) or immobilization (IMMO-1x, IMMO-3x) on day 3. Error bars represent the standard error of the mean.

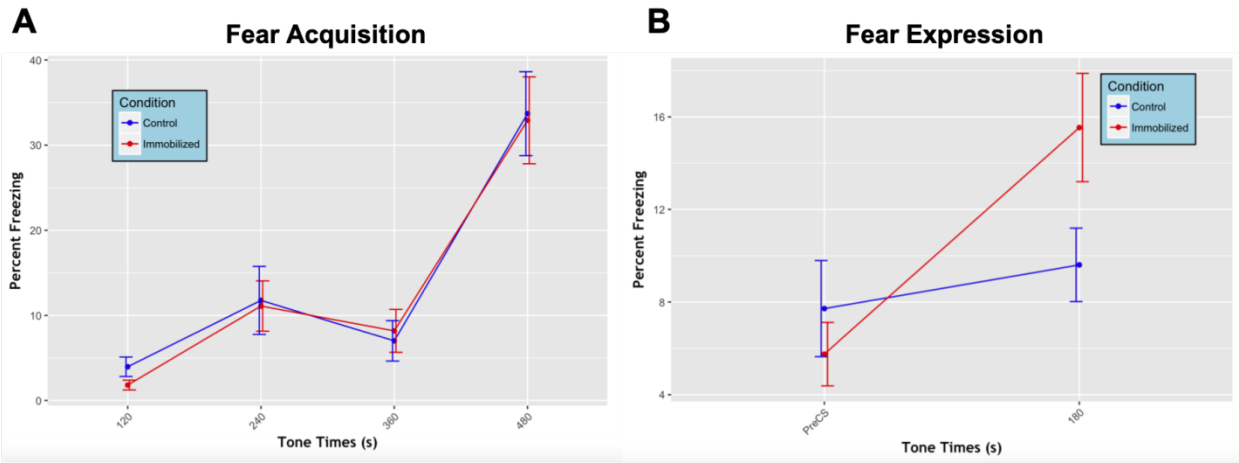

Figure 5. Effects of 3X immobilization stress on fear learning using a 50% contingency paradigm. (a) Fear acquisition with freezing at the times of tone presentation represented. (b) Fear expression with freezing across the entire tone presentation binned into one group. Error bars represent the standard error of the mean.

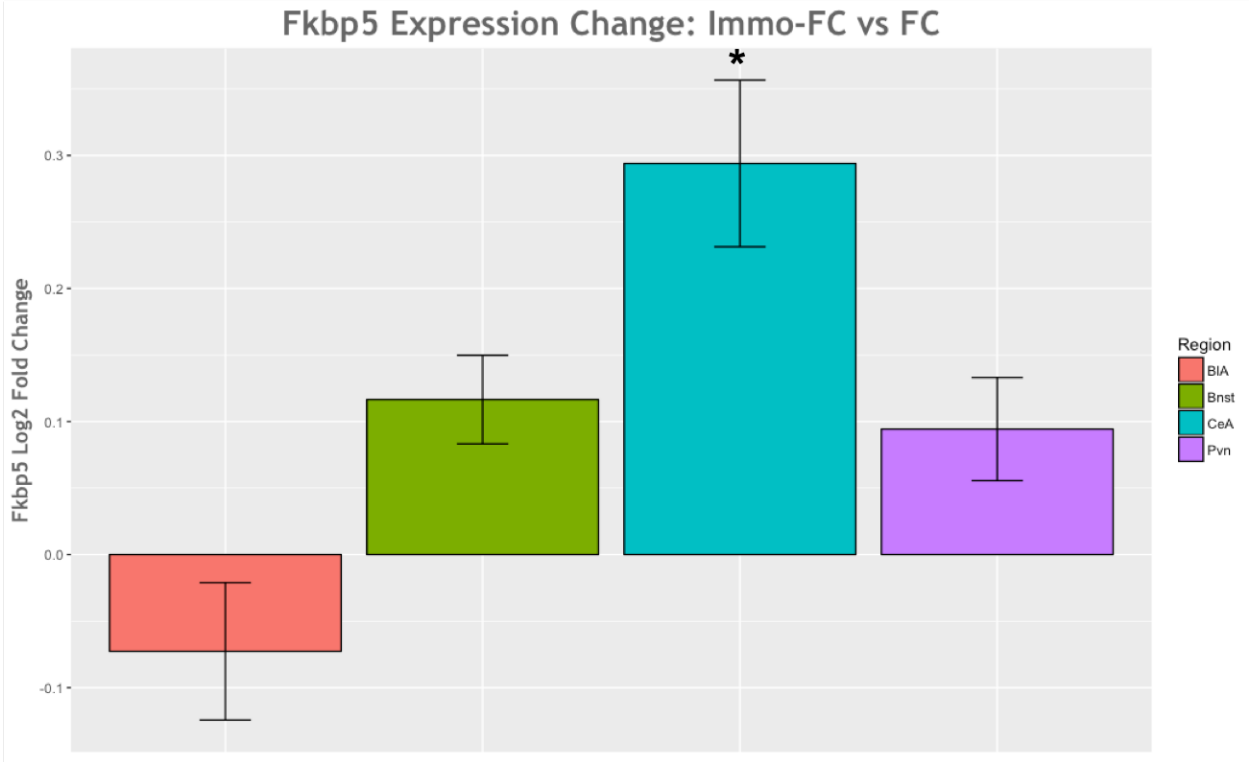

Figure 6. Expression alteration of *Fkbp5* in the BLA, Bnst, CeA, and the Pvn 2 hours after fear conditioning in mice with an immobilization history compared to control handled mice. Error bars represent the standard error of the mean.

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Chapter 4: Targeted Profiling and Epigenome Editing of *Fkbp5*

Introduction

Overall, the corpus of the literature focusing on DNA methylation, learning, and stress, suggest that it plays a crucial role in all of these processes in the CNS. However, the elucidation of specific gene pathways modulated during learning and the role that specific epigenetically active loci play in regulating these genes is not well understood. The current foundation of descriptive literature and broad manipulations have not yet demonstrated how specific epigenetic modifications influence genes of interest. Testing the causal relationships between particular epigenetic marks with chromatin dynamics, gene expression, cellular phenotypes, and behavior has been made possible with the advent of genome-editing technologies. Though still in its relative infancy, studies have demonstrated that a variety of genome-targeting proteins, covalently linked with Dnmt or Tet enzymatic domains, have the capacity to alter methylation and gene expression in a targeted manner. (Liu et al., 2016; Maeder et al., 2013)

In chapter 2 of this thesis we demonstrated an unbiased approach to dissecting molecular alterations in the amygdala with fear conditioning and showed that 5hmC putatively regulates specific gene pathways during fear. However, what the field has struggled with is whether these massive amounts of epigenetic associations are *causally* related to gene regulatory processes. Here we seek to extend our unbiased investigation of methylation dynamics in fear conditioning and to test the key associations between DNA methylation and key signatures in relevant gene pathways.

Results

Targeted Bisulfite Sequencing Replicates a reduced methylation signature in intron 1 of Fkbp5

From our unbiased analysis we identified several key genes that were differentially expressed and differentially hydroxymethylated in our discovery cohort. (Table 1) We validated these using an independent fear conditioning experiment, from which we isolated RNA and DNA from the same amygdala samples. (Figure 1) While these differentially 5-cytosine hydroxymethylated regions (DHMRs) exhibited a strong signal at the genome-wide level, we sought to clarify which cytosines in particular are differentially regulated and what is the absolute change in DNA methylation at these sites. We employed targeted sequencing of amplicons derived from bisulfite treated DNA to understand the specific alterations in DNA methylation that occur at these DHMRs. For this, we co-isolated DNA and RNA from the BLA of 8 fear conditioned and 8 home cage animals, and analyzed bilateral punches from the same animal, allowing us to make per animal correlations between gene expression and DNA methylation.

To prioritize genes exhibiting significant alterations at both the transcriptional and epigenetic level, we picked the top differentially expressed, DHMR-containing genes to replicate with qPCR. (table 1) *Fkbp5*, *Fgfr1*, and *Gabrb2* replicated with the same directionality of expression change and were significantly differentially expressed in the replication cohort. We next bisulfite sequenced the DHMR loci, from the same samples for which we isolated RNA. We found multiple CHH-context cytosines in *Fkbp5* which exhibited reduced methylation with fear conditioning, consistent with our genome wide results at this site. (Figure 2a) At a per sample

level, we found that each cytosine negatively correlated with gene expression (Figures 2b and c). These data suggested that *Fkbp5* expression and methylation dynamics are consistently regulated with fear.

This finding of differential *Fkbp5* expression and methylation in mice was consistent with literature at the level of human genetic association studies that have associated *Fkbp5* methylation status with an interaction between childhood trauma burden and susceptibility to PTSD. (Binder et al., 2004, 2008; Klengel et al., 2013; Zannas & Binder, 2014) To further elucidate the relationship between methylation at these sites and *Fkbp5* expression, we employed CRISPR-Cas9 based approaches to modulate methylation at this locus.

Targeted modulation of methylation by co-transfection with dCas9 and gRNAs

In order to test the causal relationship between DNA methylation and *Fkbp5* expression, we co-transfected various guide RNAs (gRNAs) and dead-*Cas9* fused to either *Tet1* (dCas9-Tet1) or *Dnmt3a* (dCas9-Dnmt3a) catalytic domains into N2a cells – a murine neuroblastoma cell culture system. These constructs have previously been shown to strongly and robustly alter DNA methylation at loci to which they are targeted. (Liu et al., 2016) In order to rapidly test a variety of gRNAs, we initially began our screening by using gRNA casettes consisting of PCR-amplified, U6 promoter-gRNA sequence double stranded DNA blocks. (*methods*) We co-transfected dCas9 constructs with gRNA block combinations into N2a cells. (Figure 3a) Along with dCas9-Tet1 or dCas9-Dnmt3a, we co-transfected two gRNAs targeting the cytosines in *Fkbp5* that were robustly differentially methylated in prior experiments (Figure 2a, red arrows). As a control comparison, we transfected dCas9-Tet1 or dCas9-Dnmt3a with two off-target gRNAs. (methods)

We found that targeting of the robustly differentially methylated sites in *Fkbp5* altered the expression of *Fkbp5*. Indeed, we found that targeting with dCas9-Dnmt3a to increase methylation at this site reduced the expression of *Fkbp5*. Furthermore, targeting with dCas9-Tet1 to decrease methylation at the site increased the expression of *Fkbp5* (+/- 15% expression alterations, student's t-test p-value = 0.038 and 0.023, respectively). (Figure 3b) This mirrored the association between gene expression and methylation at these genic loci seen for the robustly differentially methylated sites in this gene. (Figure 2a) This suggests that the reduction of gene methylation seen at these sites was indeed functional, and could be manipulated by locus-specific targeting.

Establishment of an optimized gRNA expression system and fluorescence-activated cell sorting (FACS)

The genome-editing scope of dCas9 is limited to the number of gRNAs that can be introduced into a single cell and the capacity to reduce off-target effects of the protein. We sought to introduce an array of optimized gRNA sequences into a vector capable of multiplexed delivery of gRNAs. (Chen et al., 2013) We leveraged the polycistronic tRNA-gRNA (PTG) approach devised in Xie, et al., 2015. (K. Xie, Minkenberg, & Yang, 2015) In short, tRNA motifs were interspersed between gRNA elements, downstream of a U6 promoter. The resulting product is a polycistronic RNA, containing tRNA motifs that are recognized and cleaved by endogenous Rnase P and Z enzymes that are utilized to produce mature tRNAs in the nucleus. The result is that gRNA sequences become freed in the nucleus to interact with dCas9 proteins and direct editing at specific loci. (Figure 4)

To clone this construct, we designed dsDNA blocks containing tRNA motifs, bookended by gRNA spacer sequences. We then used a Gibson assembly approach to ligate these blocks using the spacer sequences to enforce directionality of the construct. This allowed us to combine up to 3 gRNAs into one construct. (methods) We incorporated these polycistronic constructs into a vector backbone containing a mCherry-expressing cassette to be used for downstream co-transfection analyses and FACS. To confirm that the RNA transcript is being produced by the construct, we transfected cells, isolated RNA, degraded genomic DNA, reverse transcribed a portion of the RNA, and then used qPCR to quantify each gRNA in the RNA as compared to the cDNA. The forward primer for each gRNA was specific to its unique spacer sequence, and a common reverse primer located in the scaffold sequence was used. Given the circularity of plasmid DNA, full degradation is often impossible without extreme treatments to both preserve RNA and degrade all DNA. However, we reasoned that if RNA was being produced from the PTG, then the signal obtained from quantification of cDNA should be far greater than the signal from the RNA fraction alone, which would contain as a substrate only remaining plasmid DNA. We found that for all 3 gRNAs the signal obtained from cDNA was 300 to 47,000 fold greater than the signal obtained from RNA, suggesting that the PTG construct was expressing the polycistronic construct (Figure 5). This is consistent with the initial report of the PTG construct as well. Though we expect that equimolar amounts of each gRNA will be produced, the difference in quantification is likely due in part to different binding and PCR priming efficiencies of the forward primer used for each gRNA.

In order to rule out the possibility that our differences in *Fkbp5* expression were driven by stochastic differences in the transfection conditions, we sought to use FACS to isolate cells co-transfected with dCas9 constructs labelled with eGFP and PTG constructs labelled with

mCherry. Furthermore, for our control we used the same on-target gRNAs, but used variant dCas9 constructs containing inactive Dnmt3a domains. This allowed us to ensure the effects of dCas9 constructs changing transcriptional status of *Fkbp5* were not due to steric effects by occupying the loci, but were instead due to epigenetic editing occurring at these loci. We optimized transfection and FACS conditions to isolate high-quality RNA from cells strongly co-transfected with dCas9 constructs and the PTG construct. (Figure 6) We isolated N2a cells co-transfected with dCas9-Dnmt3a (dC9D3a), or -inactive Dnmt3a (dC9D3a-IM), and a PTG construct containing two gRNAs directed at the DHMR in *Fkbp5* (the same gRNAs used in figure 3). Of note, we found *Fkbp5* expression was reduced by the combined active dCas9-Dnmt3 construct and the gRNAs, further demonstrating the causal link between targeted DNA methylation at this locus regulating decreased *Fkbp5* expression. (Figure 7, p= 0.018)

Discussion

Herein we demonstrate an approach to the identification of dynamically modulated epigenetic loci, that are causal drivers of gene expression, from genome-wide profiling of DNA methylation. We find intronic, CHH context cytosines in *Fkbp5* that are causal drivers of *Fkbp5* expression. In the last decade, the association between CHH cytosine and gene expression has become increasingly appreciated at a genome wide level. In particular, studies of epigenetic dynamics in the central nervous system have shown these cells harbor especially dynamic CHH cytosines. (Guo et al., 2011, 2014; Yu et al., 2012) However, we believe this is the first demonstration that these cytosines are indeed driving significant alterations in gene expression in neuronal cells.

In addition, the approach of prioritizing gene pathways based on genome-wide data is particularly important. The advent of sequencing and exponential reduction in sequencing and computing costs per unit have meant that profiling of epigenetic and transcriptomic dynamics has exploded. However, in order to target specific, relevant nodes requires both an intelligent parsing of these datasets and causal testing of these targets in relevant biological contexts. This has tremendous potential to help us understand the contribution of specific, differentially epigenetically regulated loci.

Finally, it is interesting that from our unbiased approach to understanding the molecular circuitry of fear, a gene with a long history in the study of psychiatric illness emerges, *Fkbp5*. (Appel et al., 2011; Bevilacqua et al., 2012; Binder et al., 2004, 2008; Boscarino et al., 2012; Collip et al., 2013; Hartmann et al., 2012; Hubler & Scammell, 2004; Ke et al., 2018; Klengel et al., 2013; Lavebratt, Aberg, Sjoholm, & Forsell, 2010; Lee et al., 2011; Roy et al., 2010; Scammell et al., 2001; Scharf et al., 2011; Touma et al., 2011; Xie et al., 2010; Zannas & Binder, 2014; Zimmermann et al., 2011) This work further highlights the role that this gene plays in the processing of stress, fear, and anxiety, and points to the specific, causal regulation of this gene in the BLA with fear conditioning.

Tables and Figures

Gene	Discovery RNAseq (l2fc)	Replication qPCR (l2fc)	Replication p-value
gabrb2	-0.28	-0.61	0.01
fgfr1	0.28	0.42	0.02
fkbp5	0.25	0.29	0.04
lphn3	-0.3	0.42	0.04
slc10a4	-1.35	-0.75	0.08
prkcd	0.44	-0.67	0.11
kcng3	-3.04	-0.44	0.11
plekhg4	0.58	0.46	0.13
prss23	0.44	-0.49	0.19
lnpep	-2.89	-0.16	0.21
dgkh	-1.97	0.48	0.03
ptar1	-2.3	-0.24	0.35
rora	-2	-0.12	0.54
mmp16	-0.91	0.16	0.55
xrn1	-1.5	0.04	0.84
xkr4	-1.99	0.04	0.89
xpo4	-1.04	0	0.99

Table 1. Genes identified to be differentially expressed and differentially hydroxymethylated In during fear memory consolidation in the basolateral amygdala. L2fc is the log2 of the fold change as measured by either mRNA-sequencing (RNAseq) or qPCR in a replication cohort. Pval-qPCR is the Student's t-test p-value from the qPCR analysis.

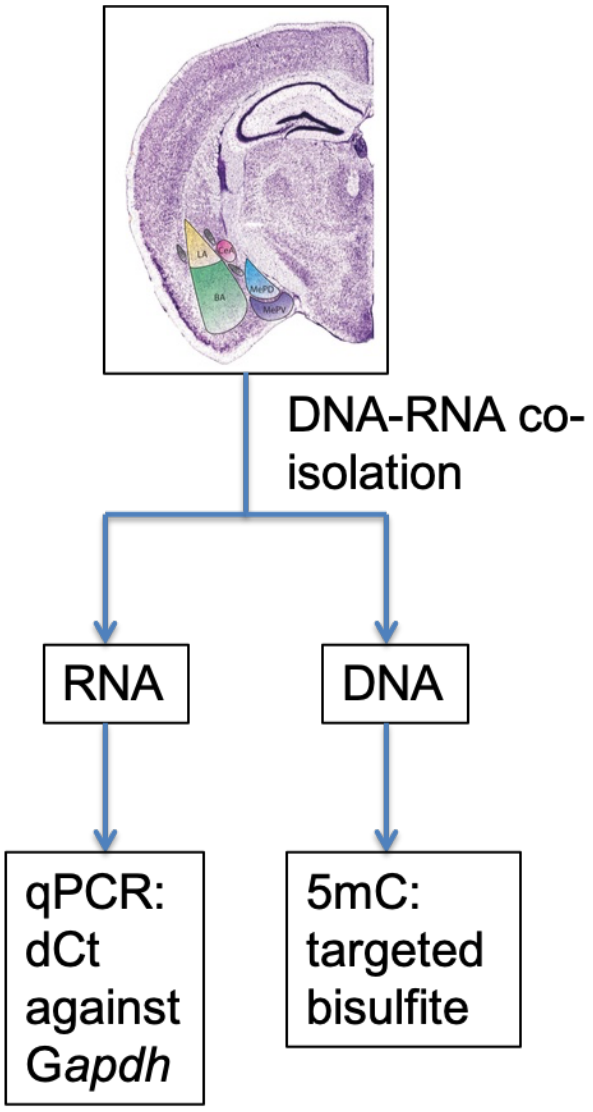


Figure 1. Schematic representation of the tissue isolation, and approach to combined analysis of mRNA and DNA methylation.

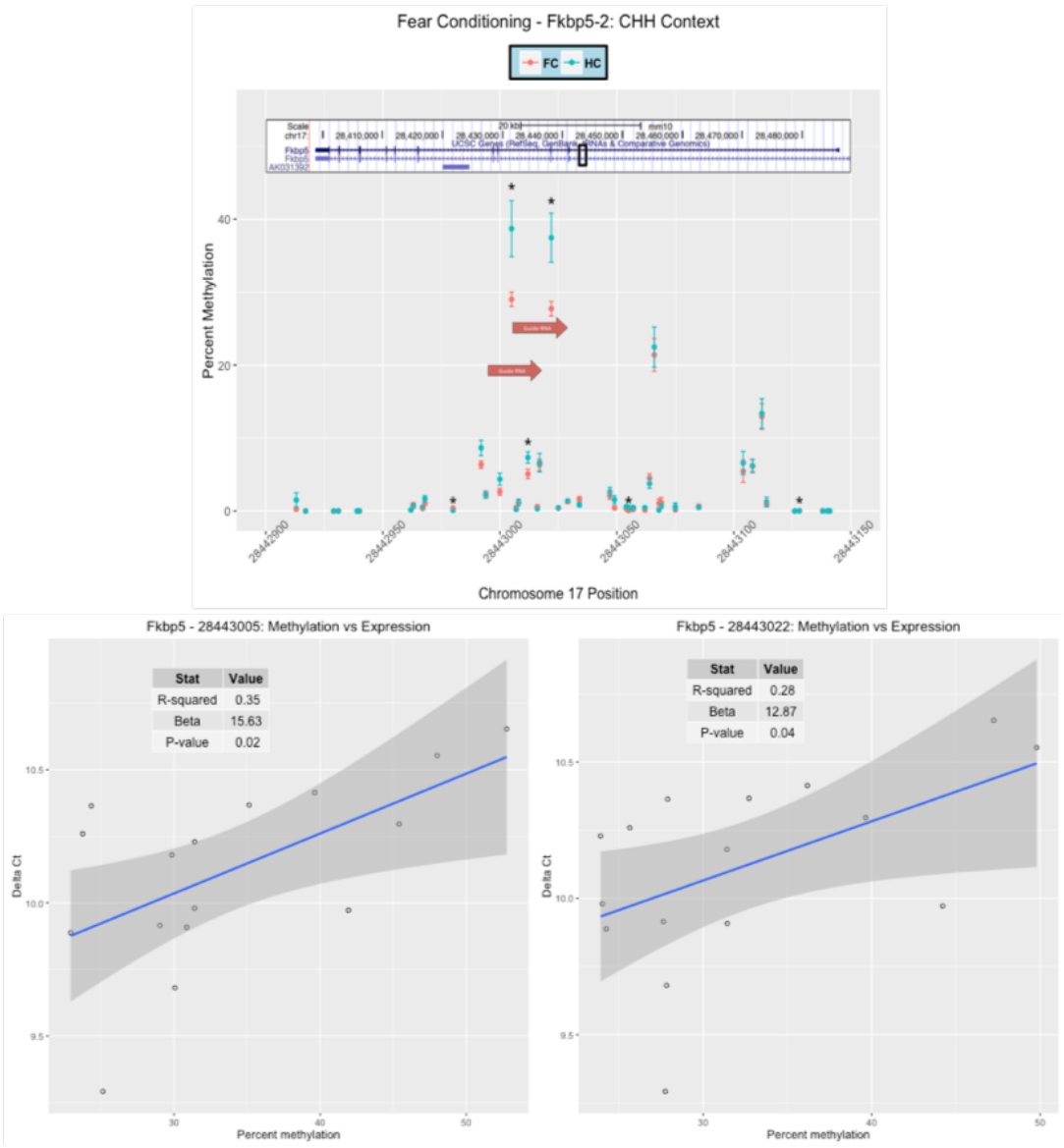


Figure 2. Results from targeted bisulfite sequencing of one fear conditioning associated DHMR in *Fkbp5*. (a) Group level differences in methylation at each CHH context cytosine in the locus. Mean is represented by the dot and the error bars are the standard error of the mean. Red dots represent the methylation in fear conditioning and blue dots the methylation in home cage. (b and c) Individual sample *Fkbp5* mRNA expression (represented as the delta ct value compared to *Gapdh*) compared to the percent DNA methylation of the two most highly methylated cytosines from (a) (cytosines above the red arrows). Error bars represent the standard error of the mean.

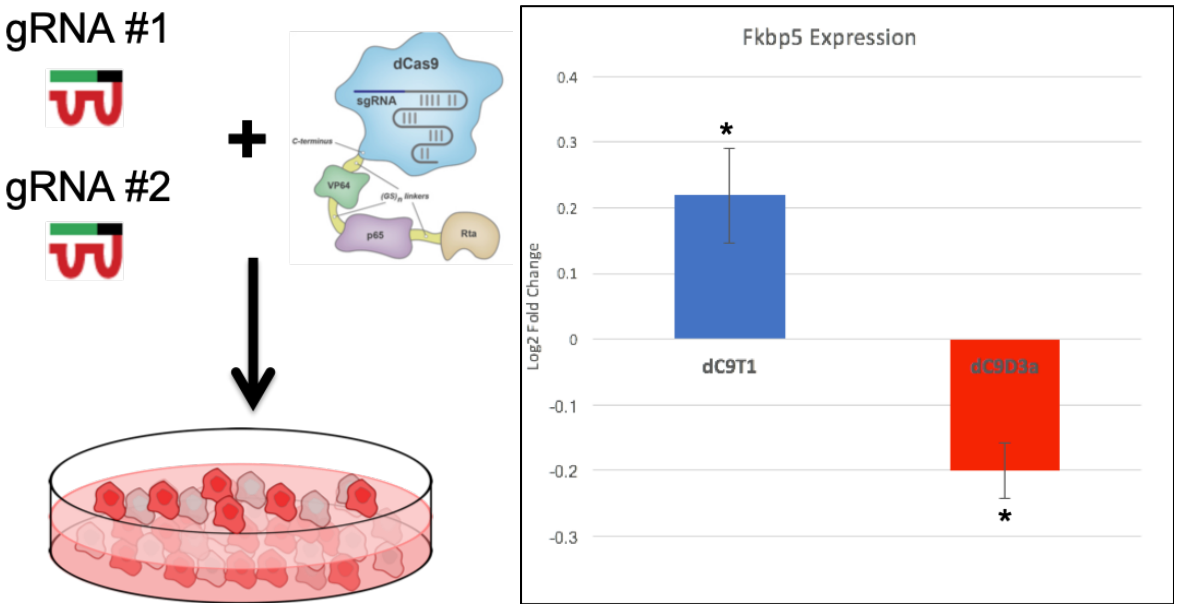


Figure 3. (a) Schematic diagram of the co-transfection of multiple gRNAs, in the form of small gRNA expression casettes (U6 promoter and individual gRNA) and dCas9 fused to an effector domain – here either *Tet1* or *Dnmt3a* catalytic domains. (b) Log2 of the fold change of *Fkbp5* expression when transfected with dCas9-Tet1 catalytic domain (blue) or dCas9-Dnmt3a catalytic domain (red) plus 2 gRNAs targeted the *Fkbp5* differentially methylated region (Figure 2a – red arrows). Error bars represent the standard error of the mean.

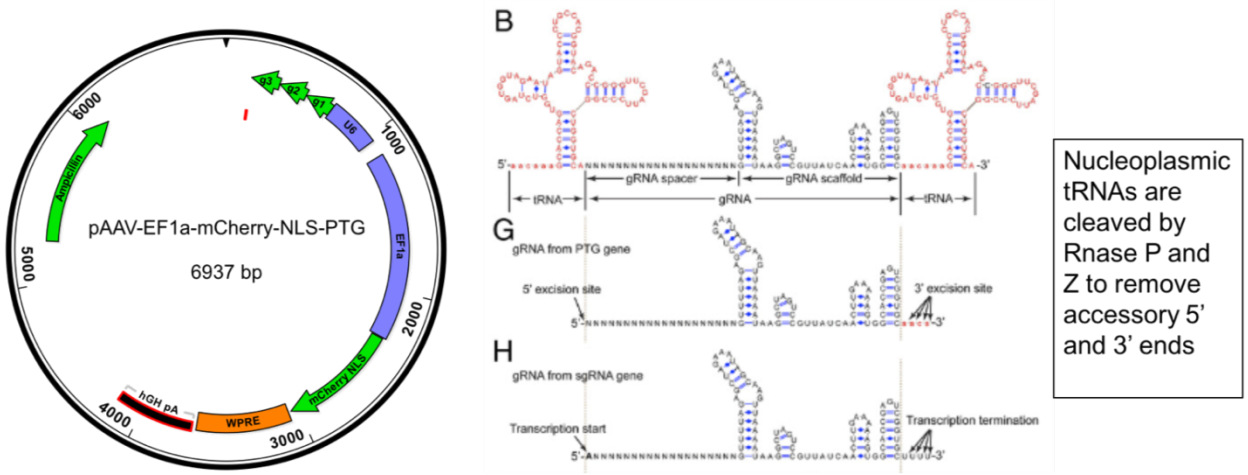
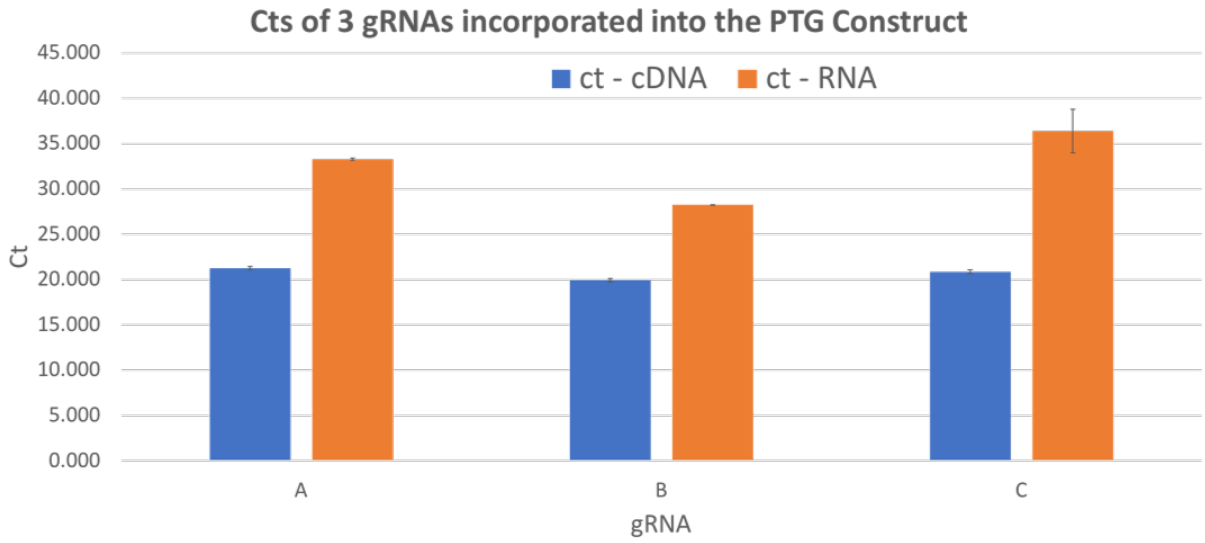


Figure 4. (a) Polycistronic tRNA-gRNA (PTG) construct, co-expressing mCherry. (b) Schematic of the PTG construct, demonstrating the specific sites of tRNA cleavage by endogenous Rnase enzymes.



gRNA	ct - cDNA	ct - RNA	dCt	fold
A	21.245	33.271	-12.025	4,169.04
B	19.948	28.238	-8.290	313.03
C	20.858	36.383	-15.524	47,129.18

Figure 5. Confirmation of RNA expression from the PTG construct. (a) Ct values of each gRNA (A,B,C) in the cDNA as compared to the RNA isolated (*only without reverse transcription*) from N2a cells transfected with the PTG expression construct. (b) Fold change calculations of each gRNA. All comparisons had p-values < 1e-6. Error bars represent the standard error of the mean.

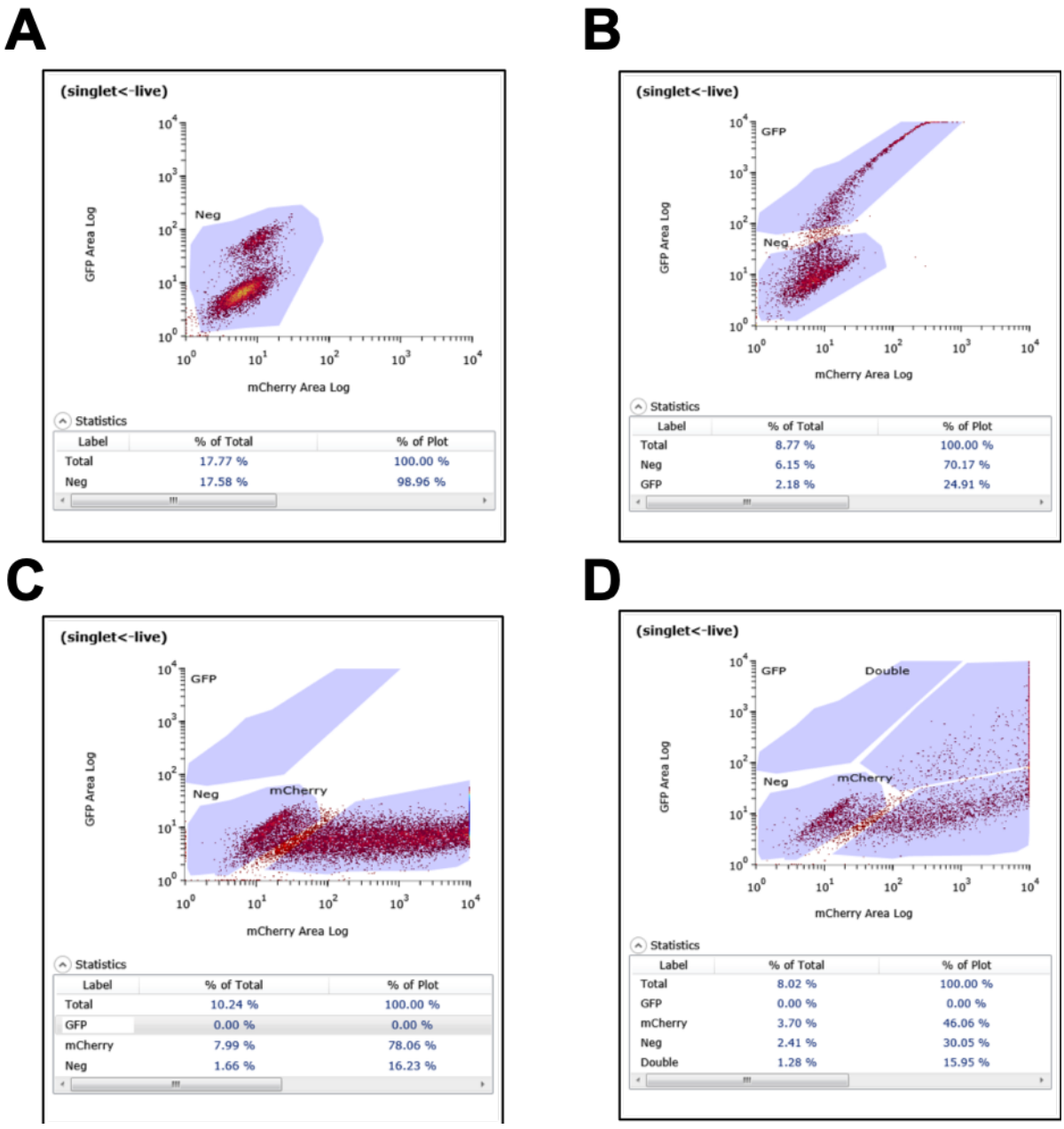


Figure 6. FACS gating of cells co-transfected with dCas9 constructs and PTG constructs. Gates were set through an iterative process of establishing the fluorescence profile of (a) untransfected cells, (b) eGFP containing dCas9 constructs, (c) mCherry containing PTG constructs and finally (d) highly co-transfected cells were identified and sorted into Trizol LS for RNA isolation.

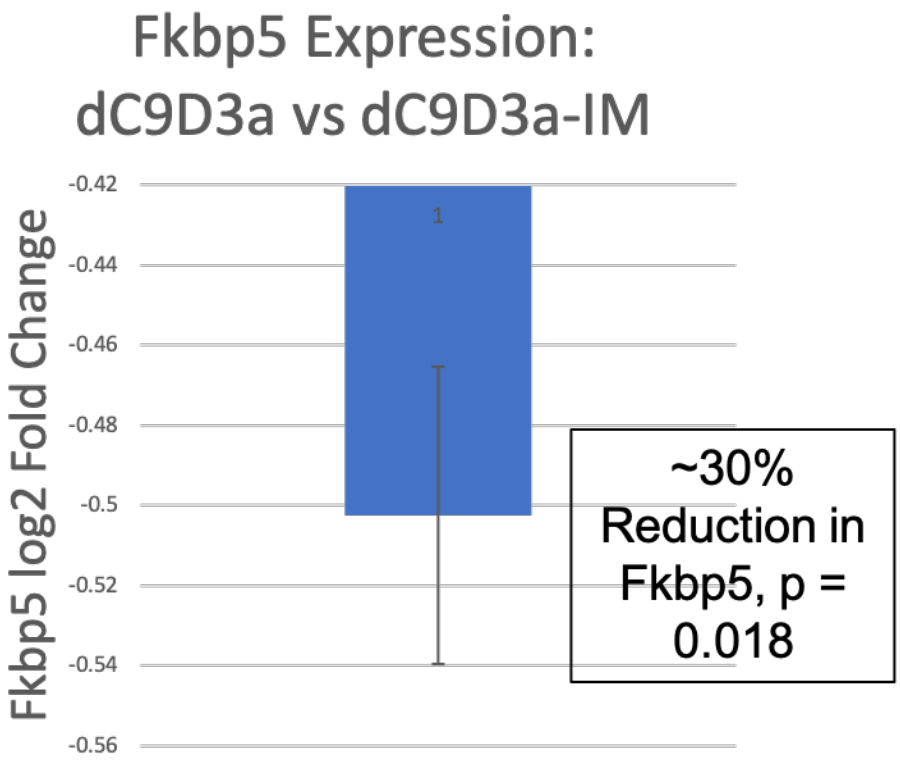


Figure 7. *Fkbp5* expression modulation by the co-transfection and FACS isolation of N2a cells expressing dCas9-Dnmta3a (or inactive Dnmt3a) and a PTG construct encoding gRNAs targeting *Fkbp5*. We find a 30% reduction in *Fkbp5* expression in the active Dnmt3a construct compared to the inactive, supporting a role for targeted DNA methylation in decreasing *Fkbp5* expression. Error bars represent the standard error of the mean.

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Discussion

Chapter 2: Epigenetic and transcriptomic profiling of the BLA during fear memory consolidation

Findings and Implications

Herein we demonstrate a methodology to identify, in an unbiased way, the gene pathways that are regulated at a transcriptional and epigenetic level in the basolateral amygdala, and key genetic loci that putatively drive the expression these genes. Broadly, we generated two distinct sets of information – what genes and gene pathways are differentially regulated during fear memory consolidation, and which methylation regulated loci in the genome are active during fear memory consolidation. The choice of profiling 5hmC allowed us to generate a novel dataset and identify loci that were potentially undergoing dynamic alterations in methylation. Previous work has demonstrated that DNA methylation may be a more dynamic and informative marker of epigenetic regulation during plasticity processes, and a wide array of literature has demonstrated that inhibiting dynamic methylation casually influences learning. (Day et al., 2013; Halder et al., 2016; Kaas et al., 2013; Levenson & Sweatt, 2005; Miller & Sweatt, 2007; Zovkic, Guzman-Karlsson, & Sweatt, 2013) This thesis aims to integrate a well-powered dataset of transcriptional events with dynamic 5hmC to clarify which biological processes are affected by the epigenetic regulation of fear.

The imperative of a cell in a biological context is to carry out a function and any single genes participate in "modules" of function within that cell. (Hartwell, Hopfield, Leibler, & Murray, 1999) The computational nature of a cell is such that it has specific inputs and outputs, and changes in gene expression are likely reflected in alterations to proteins and functional

RNAs that ultimately impact the way a cell processes information and communicates with other cells. In our studies, we captured a broad array of cells in the BLA to understand the transcriptional dynamics that underlie the plasticity process. We employed several statistical methodologies to try to understand which processes in these cells are being altered by conditioned fear. Our understanding of the 23,000 or so traditional genes in the genome is much more sophisticated than that of non-coding elements. To this end we employed the GSEA algorithm and the GO annotation database to try and understand the functional modules of molecular function altered during the process of fear memory consolidation. This revealed a series of interesting cellular functions that were altered by the plasticity process, many of which were interrelated, allowing us to home in on more general classes of molecular function. The regulation of the heat shock pathway stood out to us from this analysis, as many of the genes in this pathway have been implicated in genetic association studies of PTSD, and the role of stress hormones in regulating conditioned fear has been an active area of investigation for decades. Using our convergent datasets, we were able to parse apart this pathway and identify gene nodes that were potentially regulated by DNA methylation.

At the level of 5hmC, the calculation of 5hmC dynamics over non-coding elements – particularly enhancers, CTCF sites, and repetitive DNA pointed towards broad roles in regulating the function of candidate genes. At a translational level, many of the SNPs implicated in human disease lie in this non-coding genome. Understanding the principles of gene regulation during fear memory formation will further our understanding of the non-coding elements that are engaged during stress and fear-related plasticity – molecular circuitry that may well be conserved across mouse and man.

Limitations and Future Directions

At the level of genome-wide profiling, a difficult question is how many replicates need to be sequenced, and what resolution method should be employed. Replicates speak to the question of biological variability. Behavioral experiments, in particular with C57BL/6 mice, are very variable, and often require very large sample sizes to capture the full scope of biological variability. We chose 6 pooled samples, comprising 12 total mice, somewhat arbitrarily, based on our attempt to optimize both power and feasibility, but it is unclear at what level variability has been saturated, nor what the optimal power is for these kinds of studies. In terms of the resolution of approach, sequencing-based technologies can also be scaled by the depth of sequencing and the technique used. For instance, we used a methylated DNA capture method, and we could have sequenced these samples to a much greater depth. It is not clear to what extent more fine-grained differences would be captured with increased sequencing depth.

Another important limitation with annotation of genes and genomic elements is the richness of the annotation databases. In particular, the functions of specific groups of genes in regulating particular biological functions is a constantly evolving space, and often annotation databases are not updated to reflect current state nor are they optimized for understanding the functions of genes in particular contexts (i.e. neurons vs other cell types). This annotation problem is particularly problematic when it comes to the non-protein coding genome. A major limitation of these data is our lack of understanding of what these elements do. The relationships between enhancers and gene regulation is broadly understood, but specific targets for any given enhancer are largely unknown. Similarly, CTCF sites are thought to regulate the 3D structure of

chromatin – however, high resolution maps and maps of dynamic alterations in chromatin conformation in neuronal plasticity are not well understood. Non-coding and repetitive RNAs are another major black box – with much work needed to understand how they function at a basic level to regulate cell function. Dissecting the functions of specific non-coding elements in a variety of biological contexts will be a rich area of investigation for molecular geneticists for years to come.

Chapter 3: Behavioral model of a Stress x Fear Interaction

Findings and Implications

One of the most interesting aspects of *Fkbp5*'s function is its ability to regulate HPA axis signaling intracellularly, and the observation that it seems to be a marker of stress reactivity and traumatic history. This is in line with a plethora of evidence implicating HPA axis functioning in PTSD. This led us to develop and validate a robust behavioral model of a stress X fear interaction. Using immobilization stress spaced by 24 hours, over the course of 3 days, our protocol results in a strong anxiogenic response and a significant enhancement of fear expression after auditory fear conditioning. Furthermore, we find that *Fkbp5* expression in the CeA appears to reflect this history of stress through its expression dynamics – animals with a history of restraint stress exhibited significantly greater *Fkbp5* expression after fear conditioning than control fear conditioned animals. Finally, our validation of this robust, and easy to implement model in the hands of multiple experimenters means that it is easily accessible to many investigators. This model of stress-enhanced fear learning offers a useful platform on which we can better understand the relationship of stress with fear memory and its related molecular correlates.

Limitations and Future Directions

The utilization of animal models is a space that is currently being re-cast, in studies of behavioral neuroscience. In large part, this is driven by the failure of many behavioral pharmacology experiments in the mouse to translate to effective therapies. In general, the field of psychiatry has not seen the development of novel therapies derived from deep biological principle. SSRI's, the prototypical case of rational drug design, were identified incidentally from the "side effect" of euphoria observed in patients receiving isoniazid, an antitubercular agent that inhibited mono-amine oxidase and increased serotonin levels in the brain. This doesn't indicate that this task is impossible, and the community has taken several important steps to better define the end-goals for the study of animal models and strategies to identify therapies for disease. The first major shift is that animal models should be queried appropriately and should not simply be viewed as drug discovery platforms for the disorders they are meant to represent. (Nestler & Hyman, 2010) The second is the development of the RDoC framework of disease to better understand the biological systems altered in psychiatric diseases – i.e. rather than treating PTSD as a monolith, work to understand the specific psychological systems dysregulated in patients, such as learned fear, sleep disturbances, sympathetic arousal, etc.

Our animal model of stress-enhanced fear learning reflects the impact of stress on many of the negative valence systems dysregulated in PTSD. More specifically, we find enhanced fear memory consolidation, increased fear generalization, and increased activity of *Fkbp5*, key HPA-axis regulator in the brain. *Fkbp5* expression has been identified as a key marker of peripheral stress regulation in humans as well. (Le-Niculescu et al., 2019; Menke et al., 2012) Understanding the molecular and circuit level interactions between stress history and

psychological outputs will better inform our understanding of how traumatic history can fundamentally alter neural systems in the brain.

Chapter 4: Targeted Profiling *Fkbp5* in stress-enhanced fear learning

Findings and Implications

Our tools for understanding the genome-wide dynamics of cell regulation have exponentially advanced through the application of high-throughput sequencing to clever molecular biology techniques to enrich nucleic acids of interest. However, a major next step in our understanding of the epigenetic regulation of the genome is the application of targeted editing strategies to specific loci. Herein, we present a strategy to move forward from whole genome methylome data and identify phenotype-specific methylation dynamics, that can be tested for causality using targeted editing strategies. Using nucleotide-level profiling approaches, we delineated the specific differentially modulated cytosines driving the granular 5hmC-seq signal identified in our genome-wide screen of fear memory consolidation, and we identified key CHH context cytosines in a candidate gene that survive this analysis, *Fkbp5*. We further probe these cytosines and demonstrate a causal association between methylation activity at this locus and *Fkbp5* expression. Finally, we develop a platform for multiplexed gRNA expression, and validate our finding using a high-sensitivity FACS sorting assay.

This finding demonstrates how genome-wide data can be used not only to generate a broad understanding of molecular function, but can be used to identify specific modules of gene function that are relevant to the fear learning process. Identifying the causal associations between gene regulation and epigenetic dynamics is of crucial importance to understanding how genes are regulated by specific epigenetic changes. The regulation of gene function occurs at many levels and the increasing array of epigenome editing tools will allow us to probe the causal

relationships between non-coding elements and genome function in a huge variety of ways – including 3D conformation, enhancer-promoter relationships, DNA and histone modifications, alternative gene splicing, and others. Our study represents the first instance of an unbiased, genome-wide identification of a differentially regulated and methylated targed in fear conditioning, wherein the causal relationship between that methylation event and regulation of that gene has been established by targeted epigenome editing.

Limitations and Future Directions

One of the major limitations of our epigenetic editing work is the lack of *in vivo* evidence for the causal relationship between methylation at our fear conditioning modulated cytosines and *Fkbp5* function. The large size of the dCas9 construct and the need to co-infect with a gRNA expression construct means that a special delivery system will be needed in order to epigenetically manipulate *Fkbp5* in the brain. For example, the large carrying capacity of HSV makes it a potentially attractive vector for delivering both Cas9 constructs and larger gRNA cassettes within the same infected cells in future studies.

Another major limitation is the size of the discovery dataset. Statistical methods to identify highly differentially expressed and methylated loci now exist – however, a more well-powered discovery dataset might allow a greater number of high-confidence, dynamically methylated loci to be identified.

Conclusion

While this thesis is quite broad, the work presented here is intended to encompass strategies to utilize genome-wide information to converge on the identification of phenotype-relevant targets, the application of genome-editing tools to test the causal relationships of those targets, and the development of a robust animal model to study the impacts of stress on neurobiology. The inaccessibility and complexity the of central nervous system have made unbiased approaches, both in animal models and human studies, crucial windows to disease biology. The goal of this thesis was to use convergent, unbiased approaches to find common ground in animal models of PTSD and genetic pathways implicated in PTSD.

Through our screen of molecular dynamics during fear memory consolidation, the identification of *Fkbp5* represents another piece of evidence linking this interesting gene to the regulation of stress. Indeed, previous work has found an association between allele-specific methylation of *Fkbp5*, childhood maltreatment, and PTSD risk – mediated, in part, through long-range interactions of the intronic GRE and the promoter of *Fkbp5*. (Figure 1) (Klengel et al., 2013) We present the identification of differentially methylated CHH-context cytosines that participate in driving the methylation-directed regulation of *Fkbp5* in the amygdala (where much of the associative learning for fear occurs), during fear memory consolidation. This work demonstrates the dynamic nature of this gene, not just in the context of stress, but also in the normal physiology of fear learning.

In terms of understanding disease, elucidating the impact of specific epigenetic dynamics in context of disease-relevant biological pathways is crucial. In particular, the interaction between fear, stress, and epigenetic regulation is one that has received a great deal of attention. It would seem that key genes, such as *Fkbp5*, in the regulation of the HPA axis are relevant to psychological systems dysregulated in PTSD. In particular, it is thought that stress can have long-lasting impacts on molecular pathways – mediated in part through persistent DNA methylation events, and that these lasting epigenetic changes may contribute to disease states. (Figure 2) In terms of *Fkbp5*, the regulation of this gene and the epigenetic status of it seems to record a history of trauma and may influence risk for disease. Thus, the development of a stress x fear interaction mouse model that is robust, reproducible, and scalable offers an important starting point to understand the long-term effects of stress at the genetic and epigenetic level.

The specificity with which we can probe biology is ever increasing. Single cell transcriptional and epigenetic profiles can now be generated from thousands of cells. As always, the technological advances in biology are increasing the resolution by which we can understand molecular dynamics. The power of animal models is that we can carry out controlled experiments to investigate the neurobiological consequences of meaningful environmental and genetic perturbations – such as a history of trauma, or epigenetic editing of an HPA-axis gene, and we can ask what molecular dynamics are casually related to the readouts we have of these perturbations. Our goal as translational biomedical researchers is to utilize animal models to (1) increase our fundamental knowledge about the biological processes that drive normal and disease physiology, (2) bridge the gaps between what we understand about the human biology of disease and (3) identify novel strategies to diagnose and treat disease, and improve the quality of life of our patients. Human genetics studies are aggregating tens to hundreds of thousands of samples to

understand the genetic variants that are associated with disease. This will offer an unprecedented window into the genetic variants associated with complex neuropsychiatric disease and will help us to understand the pathological processes dysregulated in patients with PTSD. By understanding the genes and gene pathways associated with disease in humans, we can use animal models to ask causal questions about these components and better understand the biological underpinnings of stress- and anxiety-related disorders.

Combined Future Directions

One of the broad goals of this thesis was to establish a foundation for the application of unbiased genomic techniques and epigenome editing technologies to the study of translational models of PTSD. To this end, I would propose the following experimental scheme as immediate follow-up work to my thesis.

In PTSD, stressful, traumatic experiences incite a host of downstream consequences. A large swath of data points towards the heat shock complex, HPA axis regulation, and *Fkbp5* as significantly associated with PTSD and stress-related disorders. What differentiates *Fkbp5* from other targets, is the associated of long-term trauma and the epigenetic state of the gene. However, the association between persistent alterations in methylation, at any locus, and phenotypic readouts has not been rigorously tested *in vivo*. I propose the following sets of experiments to address this gap:

- Profiling *Fkbp5* expression and methylation dynamics in key brain structures in a stress-induced paradigm.
    - Social defeat
    - Stress-potentiated fear learning
    - Stress enhanced alcohol drinking or drug self-administration
    - Brain Regions:
        - CeA, Pvn, Nucleus Accumbens, dorsal and ventral hippocampus, Bnst
    - Hypothesis: a history of stress will potentiate *Fkbp5* expression, due to persistent epigenetic differences; as I have showed with stress-potentiated fear learning and CeA *Fkbp5*.
- Identifying any isoform specific differences or differential methylation of *Fkbp5* in those brain regions
    - Specific isoform differences should be taken into account when designing qPCR primers to evaluate *Fkbp5* expression changes.

- Designing gRNAs to target these regions (including specific differentially methylated regions) and incorporation of these into HSV vectors. Production of HSV vectors encoded dCas9 methylation modulators (Dnmt3a or Tet1).
- Co-infection of gRNA and dCas9 HSVs into brain regions to transiently alter the methylation of significant loci in *Fkbp5* to see if targeted methylation effects can mimic the effects of stress, or can reverse them.
- Alternative – this entire scheme can be recapitulated using genome-wide datasets to select differentially methylated sites in an unbiased fashion. This also presents the opportunity to see if heat shock pathways are also strongly upregulated by stress in multiple brain regions. This would be analogous to my chapter 2 – but using a stress-paradigm as the foundation for the genome-wide data collection. This would also offer the opportunity to identify gene modules that respond to a history of stress rather than the immediate stress of fear conditioning alone.

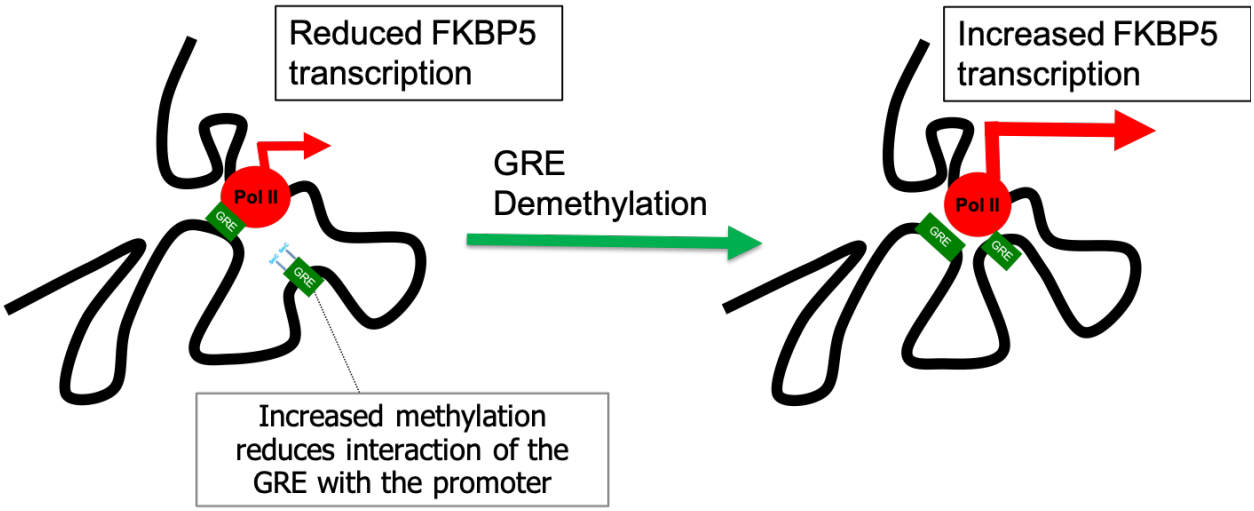

Figure 1. Mechanistic model of long-range interactions between intronic GREs and the *Fkbp5* promoter driving the regulation of gene expression. *Modified from Sharma, et al., 2015.* (Sharma, Powers, Bradley, & Ressler, 2015)

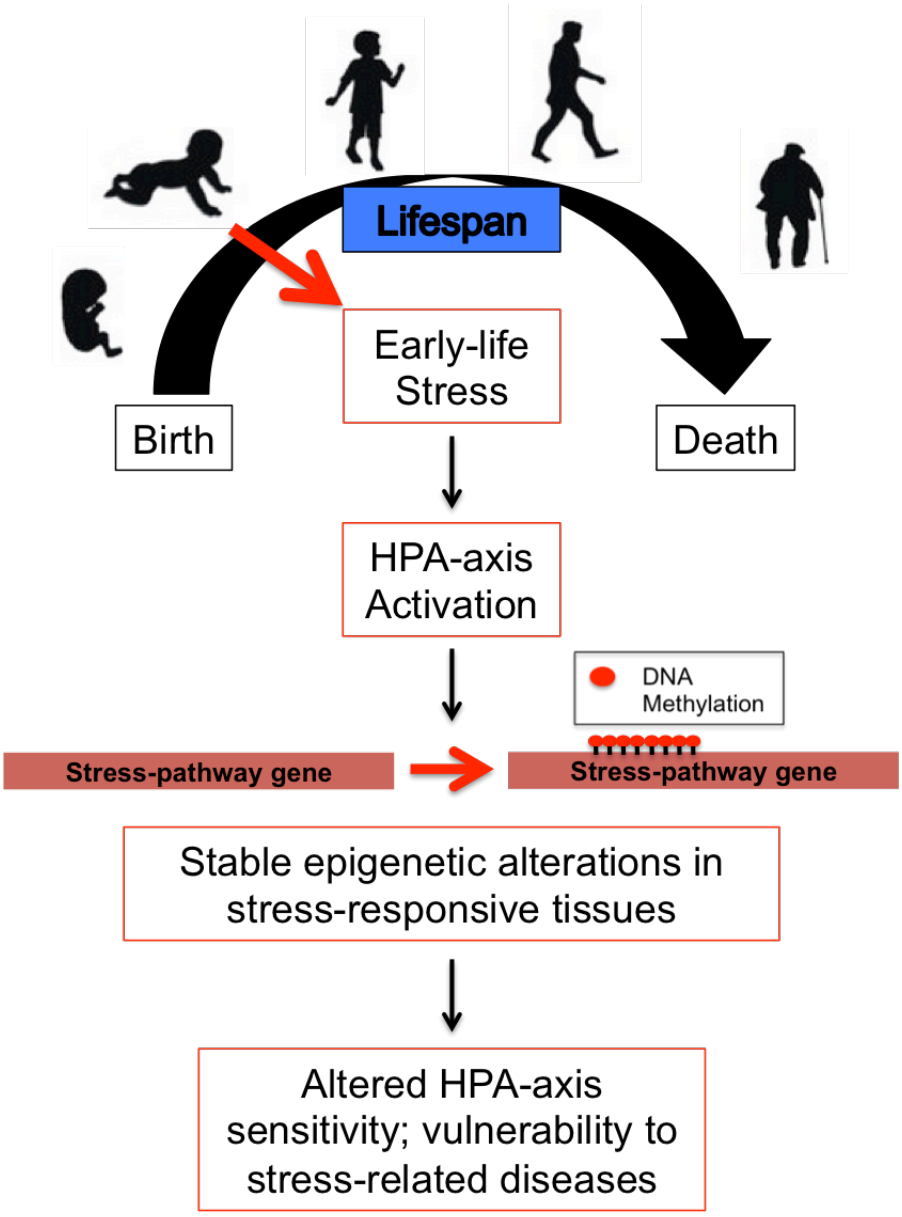

Figure 2. Illustration of the impact early life stress may have on the epigenetic regulation of stress-axis genes, leading to future pathology. *Reproduced from Sharma, et al., 2015.* (Sharma et al., 2015)

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